ABSTRACT

Title of Document: REPRODUCTIVE ISOLATION AND X CHROMOSOME MEIOTIC DRIVE IN *CYRTODIOPSIS* STALK-EYED FLIES.

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Haldane's rule states that when one sex of hybrids shows sterility or inviability it tends to be the heterogametic sex. This pattern is considered a fundamental law of speciation, and is known to be caused by several separate mechanisms. One such mechanism may be the divergence at loci affecting sex chromosome meiotic drive. *Cyrtodiopsis dalmanni* and *C. whitei* are sister species of stalk-eyed fly found in Southeast Asia and in which a sizable proportion of wild-caught males produce female-biased broods due to X chromosome meiotic drive. I cross multiple populations of these two species and use QTL mapping and DNA sequencing techniques to test three predictions of the meiotic drive hypothesis of Haldane's rule. In chapter 1, my results show that male hybrid sterility is the fastest-evolving form of reproductive isolation in these species, consistent with Haldane's rule. In chapter 2, I show that DNA sequence evolution is consistent with a pattern of repeated selective sweeps associated with X-linked meiotic drive. In chapter 3, I report the discovery of autosomal suppressors of

drive, a Y-linked suppressor, a cryptic (suppressed) driver, an inviability effect of drive, and several QTL for sterility and one QTL for male-biased progeny sex ratios. The suppressors and cryptic driver support the conclusion, from chapter 2, that meiotic drive and suppressors have evolved repeatedly in response to each other. The sterility and sex ratio QTL did not map to the same genetic marker interval, which is not necessarily inconsistent with the drive hypothesis if sufficient time has elapsed to allow additional sterility loci to evolve. Overall, these results provide several lines of support for the meiotic drive hypothesis of Haldane's rule, which is rapidly gaining traction among researchers in the field of speciation. This conclusion suggests that attempts should be made to identify specific genes affecting meiotic drive and male hybrid sterility in *Cyrtodiopsis*.

REPRODUCTIVE ISOLATION AND X CHROMOSOME MEIOTIC DRIVE IN *CYRTODIOPSIS* STALK-EYED FLIES.

By

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Dedication

To M. S. C. and C. S. C.

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Introduction

Background

Eighty-five years ago, the evolutionary biologist J. B. S. Haldane noted a pattern that has become known as Haldane's rule: when one sex of hybrids suffers from a greater degree of sterility or inviability than the other, it is usually the heterogametic (XY or ZW) sex (Haldane 1922). Although attributing the pattern to heterogamety has been questioned as recently as the early 1990's (Read and Nee 1991; Read and Nee 1993), Haldane's rule is widely accepted and regarded as a fundamental rule of speciation (Coyne and Orr 1989b) and holds in all animal taxa that have been studied (Orr 1997).

While many hypotheses have been put forth as possible explanations of Haldane's rule, three favored mechanisms are believed to operate in different circumstances (Coyne and Orr 2004; Presgraves and Orr 1998). These hypotheses are known as the dominance theory, the faster-male theory, and the faster-X theory. The dominance theory (Muller 1942; Orr 1993; Turelli and Orr 1995) assumes that epistatic interactions cause hybrid dysfunction (Dobzhansky-Muller incompatibilities: Dobzhansky 1937b; Muller 1940; Muller 1942; Orr 1995). Partially recessive interactions with at least one X-linked locus may be masked when the X is diploid but will be expressed in the heterogametic sex when the X is haploid. The second hypothesis, the faster-male theory (Wu et al. 1996), proposes that, due to sexual selection, intense competition for fertilizations leads to rapid divergence of genes affecting male reproductive traits. This leads to faster accumulation

of Dobzhansky-Muller incompatibilities in males, and earlier male than female sterility, in male-heterogametic systems. The faster-X hypothesis (Charlesworth et al. 1987), regarded by some as the least important of the three (Orr 1997), states that when favorable mutations are partially recessive, higher substitution of those mutations on a hemizygous chromosome lead to faster evolution on the X than on autosomes.

A fourth hypothesis for the cause of Haldane's rule, which is less widely accepted, is the meiotic drive hypothesis (Frank 1991; Hurst and Pomiankowski 1991). This hypothesis states that as sex chromosome meiotic drive systems diverge between isolated populations, heterogametic hybrids fail to complete gametogenesis, leading to sex-specific sterility or possibly inviability. After an initial period of intense criticism (Coyne et al. 1991; Coyne and Orr 1993; Johnson and Wu 1992), the drive hypothesis has emerged as a fertile area of research. Recent reinterpretations of the hypothesis point out that due to two different mechanisms of meiotic drive, the concept applies to both male- and female-heterogametic taxa (Tao and Hartl 2003).

Meiotic drive is a selfish genetic system that causes a chromosome to be overrepresented in gametes at the expense of its homolog (Sandler and Novitski 1957), and is therefore one cause of the effect known as transmission ratio distortion. The two major mechanisms of drive are called genic drive and chromosomal drive. In genic drive, a locus on one chromosome prevents transmission of sperm carrying that chromosome's homolog. It is this mechanism of drive that is hypothesized to lead to Haldane's rule in male-heterogametic species. In chromosomal drive, centromeres compete to reach the ovum during meiosis instead of being relegated to a polar body (Henikoff et al. 2001; Henikoff and Malik 2002); this mechanism of drive is hypothesized to lead to Haldane's

rule in female-heterogametic species. In genic sex chromosome drive, a locus on one sex chromosome produces skewed offspring sex ratio by eliminating viable gametes containing the other sex chromosome. In X drive, Y-bearing sperm affected by the drive allele degenerate and become greatly outnumbered by X-bearing sperm (Montchamp-Moreau and Joly 1997; Novitski 1947; Wilkinson and Sanchez 2001). The potential importance of the drive hypothesis of Haldane's rule can be seen by examining the wide distribution of meiotic drive systems in animals: numerous cases have been reported in Diptera, and examples occur in Lepidoptera, Hemiptera, guppies, rodents, birds, flowering plants, and even humans (summarized in Hurst and Pomiankowski 1991; Jaenike 2001).

Orr *et al*. (2007) have recently summarized evidence from studies of *Drosophila* that is consistent with the drive hypothesis for Haldane's rule. Cryptic drive systems have been discovered that are masked in the populations in which they occur, but which are expressed in hybrids (Dermitzakis et al. 2000; Mercot et al. 1995; Orr and Irving 2005; Tao et al. 2001; Yang et al. 2004). In addition, male hybrid sterility loci and meiotic drive-related loci have been shown to map to the same chromosomal intervals (Orr and Irving 2005), or possibly to the same locus (Tao et al. 2001). From another taxonomic kingdom, hybrid sterility QTL are associated with transmission ratio distortion in tomato (Moyle and Graham 2006). In addition to these supportive empirical results, the feasibility of the drive hypothesis has been demonstrated theoretically (Adams 2005).

Experimental Investigation of the Meiotic Drive Hypothesis

The hypothesis that divergence between sex chromosome drive systems causes Haldane's rule makes several testable predictions, four of which I investigate here. These predictions are based on the action of X chromosome drive in a male-heterogametic system; i.e. where males have one X and one Y chromosome and females have two X chromosomes. The first prediction is that male hybrid sterility, which is the only form of isolation so far to have been linked to meiotic drive, will evolve faster than all other forms of isolation. Second, if divergence between populations at meiotic drive loci is rapid enough to cause early reproductive isolation, signatures of this rapid divergence will be retained in linked DNA sequence. Third, this pattern of evolution of meiotic drive is predicted to generate multiple suppressor loci and cryptic drivers. Finally, if male hybrid sterility is caused by the same loci as meiotic drive, QTL affecting the two traits will map to the same chromosomal intervals. In the following paragraphs I briefly describe the study system and methods used to test each of these predictions.

Study System

Cyrtodiopsis dalmanni and *C. whitei* are sister species of stalk-eyed flies from the family Diopsidae (Wilkinson et al. 1998a) which are of interest for the study of several evolutionary questions. Multiple populations have been collected from these species' ranges in Southeast Asia (Christianson et al. 2005; Swallow et al. 2005) and maintained in the lab since 1999. Both species possess exaggerated, sexually dimorphic eyestalks

(Burkhardt and de la Motte 1985, Figure 1), which affect female mate choice (Burkhardt and de la Motte 1988; Wilkinson and Reillo 1994) and male competition (Panhuis and Wilkinson 1999), and eyespan is correlated with body size (Wilkinson and Dodson 1997). Both *C. dalmanni* and *C. whitei* exhibit meiotic drive (Presgraves et al. 1997), and eyespan is an indicator of males' meiotic drive status (Johns et al. 2005; Wilkinson et al. 1998b).

The various populations of *C. dalmanni* and *C. whitei* are separated by a minimum of 0.3% mitochondrial DNA sequence divergence and all population crosses feature some level of prezygotic or postzygotic isolation (Christianson et al. 2005). However, these distinct populations cannot be differentiated by eyespan allometry and therefore do not appear to be speciating as a result of sexual selection (Swallow et al. 2005). There are also no obvious ecological differences between populations. While no ecological studies have been performed on wild *Cyrtodiopsis* flies, all of the populations studied in this dissertation were collected in remarkably similar locations, and all from within a few yards of fast-moving streams (G. Wilkinson, pers. comm.). In addition, the populations have all been successfully maintained for nearly 10 years under identical laboratory conditions, eating identical pureed corn medium. Because there is little reason for concern about habitat differences between populations, and because males and females mate multiply and show little or no precopulatory courtship (Wilkinson et al. 1998a; Wilkinson et al. 2003), these species are amenable for studies of reproductive isolation. Finally, the growing infrastructure for molecular genetic research – approximately 50 microsatellites are in use (Wright et al. 2004) and thousands of

expressed sequence tags are under development (G. Wilkinson, pers. comm.) – makes this system increasingly powerful for addressing a variety of evolutionary questions.

Prediction 1: Rapid Evolution of Male Hybrid Sterility

In chapter 1, I report on a study in which I test the relative rates of evolution of several forms of reproductive isolation in *Cyrtodiopsis*. I conducted reciprocal crosses between most combinations of six *C. dalmanni* and two *C. whitei* populations and record matings and other behaviors, sperm transfer frequency, the number of progeny produced, and the proportion fertile progeny. I analyzed these data against the average mitochondrial DNA sequence divergence between populations and found a gradual decline in mating rate and sperm transfer frequency with increasing genetic distance, which indicated gradual evolution of prezygotic isolation. A gradual decline in log transformed progeny production indicated a similar pattern in the evolution of postmating isolation (excluding hybrid sterility). Female hybrid sterility appeared at a similar level of divergence as prezygotic and postmating isolation, but male hybrid sterility was present between populations with much less divergence. This result indicated, in agreement with the prediction of the meiotic drive hypothesis of Haldane's rule, that male hybrid sterility was the first form of reproductive isolation to evolve between populations of flies in this genus.

This study underscored the difficulties with using indices of reproductive isolation in meta-analyses to detect broad speciation trends (Coyne and Orr 1989a; Coyne and Orr 1997; Sasa et al. 1998; Zouros 1973). Clearly, it is impractical to test dozens of species

for several measures of reproductive isolation for every study. However, compiling multiple studies with different methodologies entails compromises that affect analytical power. For example, the postzygotic isolation index in Coyne and Orr's landmark meta-analysis of speciation in *Drosophila* ranked interspecific crosses on a scale from zero (all hybrids either sterile or inviable) to four (all hybrids both viable and fertile) (Coyne and Orr 1989a; Coyne and Orr 1997). These criteria mean that a cross which fails to produce any hybrid males will recieve the same score as a cross which yields many sterile males. Thus, the first publication of the analysis (Coyne and Orr 1989a) used an impressive array of species comparisons but failed to draw all of the conclusions that were made when the issue was revisited with a larger sample size (Coyne and Orr 1997).

Because I analyzed each type of reproductive isolation as a quantitative measure, I avoided the problems that plague meta-analyses. Instead of collecting food cups and counting progeny from a single cage containing multiple males and females, I housed each male in his own cage with three females and observed matings. My methods required more time and space, and restricted the number of population combinations and sample sizes within each comparison. However, the data I collected allowed me to make detailed comparisons between types of reproductive isolation that otherwise would not have been detected by simpler approaches.

Prediction 2: DNA Sequence Polymorphism and Divergence

In chapter 2, I examined the DNA sequence of two populations of *C. dalmanni* for evidence of recent positive selection. I sequenced autosomal and X-linked DNA

fragments and analyzed the sequences for patterns of polymorphism and divergence. I show that there is greatly reduced polymorphism associated with meiotic drive, which is consistent with the action of positive selection and hitchhiking of linked genes. I also show that the X^D and X^{ST} chromosomes have diverged significantly with respect to one another and to a greater extent than the autosomal loci. These results are consistent with antagonistic coevolution within the complex of meiotic drive-associated loci, as expected if drive is causing Haldane's rule.

Prediction 3: QTL Locations in Drive and Sterility

In chapter 3, I conducted two quantitative trait loci (QTL) studies to compare the locations of chromosomal regions affecting male hybrid sterility and X chromosome meiotic drive. I performed two crosses between the Gombak and Soraya populations of *C. dalmanni*, one with a male that expressed meiotic drive and the other with a male that did not carry drive. After two backcross generations I collected data on the fertility and progeny sex ratio of 699 male progeny and genotyped these males at 27 microsatellite markers. I failed to locate QTL affecting the two traits in the same marker interval, but I did demonstrate that both traits are affected by the same portion of the X chromosome, which is tightly linked in drive-carrying males by a large paracentric inversion. I also uncovered a cryptic drive chromosome, an autosomal suppressor of drive and a Y-linked modifier that led to severely male biased broods. These results provide mixed support for the meiotic drive hypothesis of Haldane's rule.

Before mapping sterility, I had to decide how to score the trait. I allowed the flies to mate and counted, or noted the absence of, their progeny. However, in many studies of male hybrid sterility a different measure of fertility is used. Studies of *Drosophila* fruit flies most commonly score the presence of motile sperm (e.g. Coyne 1984; Coyne and Charlesworth 1989; Coyne et al. 2004; Moehring et al. 2006). In mice, both testis weight and sperm count have been used as measures of fertility (e.g. Britton-Davidian et al. 2005). The obvious appeal of such measures is that hybrid progeny do not have to be kept alive for days or weeks after reproductive maturity, saving time and resources and allowing for greater sample sizes. And indeed, in my study I had difficulty obtaining a sufficient number of fertile males to map progeny sex ratio. But the method chosen to score fertility can have a dramatic impact on the final outcome of the study.

In both mouse and fruit fly studies, data were collected to ensure correlation between fertility as measured by progeny production and by the surrogate trait (Britton-Davidian et al. 2005; Coyne 1984; Storchova et al. 2004), and the results of the two measures do not always agree. In mice, multiple QTL have been detected for testis weight and sperm count where only a single QTL was found for progeny count (Storchova et al. 2004). And, in the study that began the tradition of using sperm motility to measure fertility in fruit flies, at least one genotype class was found to produce motile sperm but no offspring (Coyne 1984). Coyne argued that using progeny production to score fertility confounds mating ability with sperm competence and that the missing progeny were due to the males' lack of competitive ability. I argue, however, that a hybrid male that cannot produce progeny is sterile whether the reason is a sperm deficiency or incorrect mating behavior. Because I required progeny sex ratio data, as

well as fertility data, I chose to count progeny rather than score males for motile sperm since that would have required considerable additional time and ultimately reduced my sample size. I later scored (thawed) frozen testes for the presence of mature sperm bundles, and while essentially all fertile males' testes contained mature sperm bundles, only approximately one half of males with mature sperm bundles produced offspring (Table 1). In a future paper I plan to report on how QTL for sperm production and sperm length differ from QTL for male hybrid sterility.

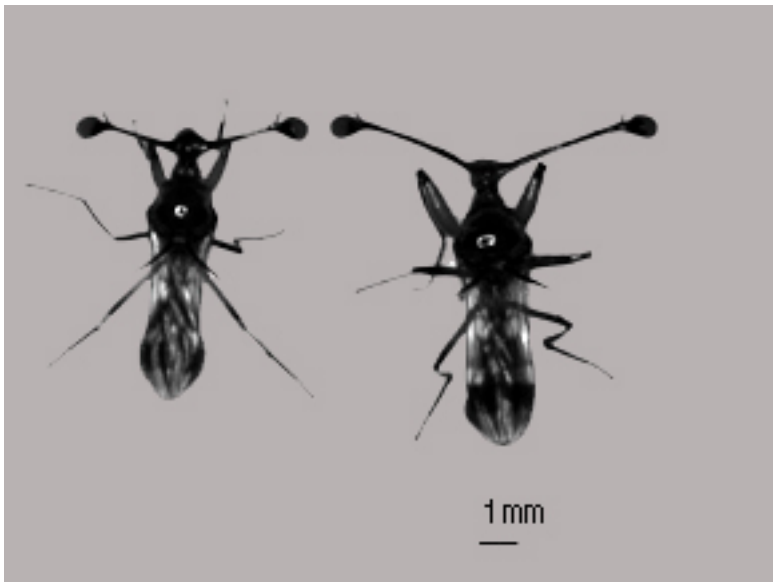
Summary of Conclusions

Results from the studies in this dissertation provide mostly positive support for the meiotic drive hypothesis of Haldane's rule. I tested three predictions made by this hypothesis, and the results here are entirely consistent with two and partially consistent with the third. I failed to find QTL affecting meiotic drive and male hybrid sterility that map to the same marker intervals, but I did find evidence for rapid evolution of male hybrid sterility, cryptic meiotic drive, autosomal and Y-linked suppressors of meiotic drive, and antagonistic coevolution between drive and suppressors. These results join the growing body of evidence that indicates the importance of meiotic drive in speciation, and show that the genus *Cyrtodiopsis* will be a valuable system in which to investigate many questions about speciation.

Table 1: The number of males with and without mature sperm bundles upon dissection. Fertile and sterile males are from all four backcross families are pooled.

	Fertile Males	Sterile Males
Mature sperm bundles present	273	197
Mature sperm bundles absent	4	214

Figure 1: Female (left) and male (right) *Cyrtodiopsis dalmanni*, illustrating their dimorphic eyestalks. Photo by A. Lorsong.



Chapter 1: Rapid Evolution of Postzygotic Isolation in Stalk-eyed Flies

Abstract

We test the relative rates of evolution of pre- and postzygotic reproductive isolation using eight populations of the sexually dimorphic stalk-eyed flies *Cyrtodiopsis dalmanni* and *C. whitei*. Flies from these populations exhibit few morphological differences yet experience strong sexual selection on male eyestalks. To measure reproductive isolation we housed one male and three female flies from within and between these populations in replicate cages and then recorded mating behavior, sperm transfer, progeny production and hybrid fertility. Using a phylogeny based on partial sequences of two mitochondrial genes, we found that premating isolation, postmating isolation prior to hybrid eclosion, and female hybrid sterility evolve gradually with respect to mitochondrial DNA sequence divergence. In contrast, male hybrid sterility evolves much more rapidly – at least twice as fast as any other form of reproductive isolation. Hybrid sterility, therefore, obeys Haldane's rule. Although some brood sex ratios were female-biased, average brood sex ratio did not covary with genetic distance, as would be expected if hybrid inviability obeyed Haldane's rule. The likelihood that forces including sexual selection and intra- and intergenomic conflict may have contributed to these patterns is discussed.

Introduction

From a genetical perspective, understanding the process of speciation amounts to understanding the process of reproductive isolation. Factors inhibiting interspecies mating (premating prezygotic isolation), interspecies fertilization (postmating prezygotic isolation or gametic isolation), and the fitness and fertility of hybrid organisms (postzygotic isolation), create barriers to gene flow that eventually lead to speciation. It can be difficult to determine the sequence of genetic changes causing each of these effects during the earliest stages of isolation because the species available for study have generally been separated for many thousands or even millions of years (Orr 1995) and may currently inhabit isolated locations. However, laboratory study of mating behavior and hybridization among multiple species or divergent populations has provided some insights into the early isolation process.

In a comprehensive meta-analysis, Coyne and Orr (1989a; 1997) describe several important features of reproductive isolation in *Drosophila*. First, allopatric species pairs tend to acquire prezygotic and postzygotic isolation at equal and constant rates. Subsequent studies on ducks (Tubaro and Lijtmaer 2002), doves (Lijtmaer et al. 2003), butterflies (Presgraves 2002), and frogs (Sasa et al. 1998), have also found evidence that postzygotic isolation evolves gradually over time, but a few studies have reached other conclusions. For example, Tilley et al. (1990) failed to detect a relationship between prezygotic isolation and allozyme differentiation in plethodontid salamanders after taking into account geographic proximity. Moyle et al. (2004) reported that reproductive

isolation correlates with genetic distance with *Silene* but not in two other angiosperm genera, possibly due to polyploidy events. Mendelson (2003) found evidence for accelerated evolution of prezygotic over postzygotic isolation in the fish genus *Etheostoma*, possibly as a result of sexual selection or the absence of degenerate sex chromosomes in these fish. However, her only measure of postzygotic isolation was hybrid inviability.

A second feature of reproductive isolation identified by Coyne and Orr (1989a; 1997) was that sympatric species pairs accumulate prezygotic, but not postzygotic, isolation more rapidly than allopatric pairs, presumably as a consequence of reinforcement (Dobzhansky 1937b). Similar results have also been reported by Marin et al. (1993), who performed mass pairwise crosses of 30 populations from 10 species of the *Drosophila repleta* group. However, Moyle et al. (2004) found no such pattern in the three angiosperm genera studied. A third feature is that Haldane's rule, that is, a bias towards hybrid sterility or inviability in the heterogametic sex (Haldane 1922), appears during the earliest stages of postzygotic isolation in male-heterogametic *Drosophila*. This result has also been supported for female-heterogametic taxa (Presgraves 2002; Price and Bouvier 2002).

A potential weakness of meta-analyses is that they typically incorporate an isolation index which compresses information about multiple characters into a single categorical metric. Studies generally combine either male and female hybrid sterility or hybrid sterility and hybrid inviability to accommodate data from a variety of experimental designs. Such an index can, for example, allow a species pair that produces many sterile male hybrids to be scored identically to a species pair that produces no male

hybrids at all. While it then may be possible to draw conclusions regarding the relative rates of evolution of prezygotic isolation to postzygotic isolation, in general, or of hybrid sterility and hybrid inviability, differences between forms of isolation that have been combined into indices could confound interpretation. For example, Coyne and Orr (1989a) at first failed to detect a difference between the rate of evolution of hybrid sterility and hybrid inviability, using a categorical description of sterility and inviability and combining male and female hybrid sterility. They were later able to detect a difference (1997), as have other studies (Presgraves 2002; Sasa et al. 1998; Wu 1992) using different analyses.

In this paper, we measure premating isolation, postmating isolation, male hybrid sterility, and female hybrid sterility for a single study system. We estimate evolutionary rates for each of these forms of reproductive isolation by using mitochondrial DNA (mtDNA) sequence divergence between allopatric populations from two *Cyrtodiopsis* species of Southeast Asian stalk-eyed flies. To avoid problems associated with isolation indices we scale measures of isolation relative to within-population measurements prior to comparing them to one another. We also test if hybrid sterility and hybrid inviability conform to Haldane's rule, that is, if male hybrids exhibit sterility and/or inviability before female hybrids.

Diopsid stalk-eyed flies in the genus *Cyrtodiopsis* are well suited for studying the evolution of reproductive isolation for several reasons. First, because these flies are easily captured in the field and can be reared in captivity (Burkhardt and de la Motte 1983; Wilkinson 2001), many types of reproductive isolation can be measured in the laboratory. Second, both males and females are promiscuous and exhibit little or no precopulatory

courtship behavior (Wilkinson et al. 1998a; Wilkinson et al. 2003). Thus, direct observations of copulation rates provide quantitative information on premating isolation. Third, while stalk-eyed flies are well-known for having sexually dimorphic eyestalks that influence both female mate choice (Wilkinson et al. 1998b) and male-male competition (Panhuis and Wilkinson 1999), little to no divergence in eyespan allometry or body size has occurred between the populations used in this study (Swallow et al. 2005). Thus, any reproductive isolation between populations is not likely to be the result of sexual selection on eyespan. Fourth, robust phylogenetic hypotheses based on DNA sequence information are available for several species in the family (Baker et al. 2001) as well as among populations of two species in the genus (this paper). These populations occur on islands in the Sunda Shelf region of Southeast Asia and vary in geographic and genetic separation, but all exhibit evidence of X chromosome meiotic drive (Wilkinson et al. 2003). Thus, they provide a natural experiment for inferring evolutionary change in reproductive isolation.

Materials and Methods

Study Populations

For this study we used six populations of *C. dalmanni* and two populations of *C. whitei* derived from flies captured in the Sunda Shelf region of Southeast Asia between January, 1996 and September, 2000 (Figure 2). Populations were established with at least

50 adult individuals and subsequently kept at higher numbers in 30 x 30 x 30 cm Plexiglas population cages in the lab. *Cyrtodiopsis dalmanni* were captured near Cameron Highlands, Malaysia (4˚ 15' N, 101˚ 21' E); near Ulu Gombak, Malaysia (3˚ 12' N, 101˚ 42' E); near the Soraya field station, Sumatra, (2˚ 52' N, 97˚ 54' E); near Bukit Lawang, Sumatra, (3˚ 35' N, 98˚ 6' E); at a forestry research station in Bogor, Java (6˚ 34' S, 106˚ 50' E); and at the Kuela Belalong Field Station in Brunei, Borneo (4°30' N, 115°10'). *Cyrtodiopsis. whitei* were captured at Ulu Gombak and near Chiang Mai, Thailand (19˚ 9' N, 98˚ 7' E). We classified flies to species based on morphological comparisons to specimens housed at the National Museum of Natural History, Washington, D.C.

In the laboratory, stock populations are maintained in a humidified chamber at 25°C with a 12:12 h light-dark cycle. Adult animals are fed twice weekly with pureed corn treated with methyl paraben to inhibit mold. Flies used in mating trials were bred from stock populations by allowing females to oviposit on 50 ml of pureed corn in 100 ml plastic cups. Larvae were kept on the same light and temperature regime as their parents. After eclosion from the cups flies were kept in single-sex cages for at least 4 weeks to ensure virginity and reproductive maturity (Lorch et al. 1993).

Phylogenetic Analysis

Phylogenetic relationships between the eight populations used in this study (Fig. 2) were inferred using partial gene sequences of two different mitochondrial genes: cytochrome oxidase II (COII) and 16S ribosomal RNA (16S). For each population we

extracted DNA from five or more field-collected flies, which had been frozen or preserved in ethanol, using Qiamp tissue extraction kits (Qiagen, Valencia, CA, USA). We then amplified the two gene fragments using primers and polymerase chain reaction (PCR) protocols optimized for diopsid flies (Baker et al. 2001). Using amplifying primers, we sequenced both strands of the products using Big Dye cycle sequencing chemistry (PE Applied Biosystems, Foster City, CA) on an ABI 310 automated genetic analyzer. These partial gene sequences can be found in GenBank (COII: AY876495-AY876545; 16S: AY876546-AY867595). Sequence data for *Diopsis apicalis* (COII, AF304777; 16S, AF304742) and *Eurydiopsis argentifera* (COII, AF304764; 16S, AF304729) were obtained from GenBank and included as outgroups to root the tree in the phylogenetic analyses.

Phylogenetic hypotheses were generated using maximum-likelihood (ML) and maximum-parsimony (MP) in Paup* 4.0b10 (Swofford 2001) on the combined COII and 16S data set. Exactly five animals per population were used for generating phylogenetic trees, but all available sequences were used for calculating genetic distances between populations. Optimal parameters of DNA substitution rates for ML searches were obtained using Modeltest 2.0 (Posada and Crandall 1998). The most appropriate model of DNA substitutions for this data set corresponded to a TIM model (Transitional model: rAC = rGT ≠ rAT = rCG ≠ rAG ≠ rCT) with a transition:tranversion (ts:tv) ratio of ~1:4.4, rate heterogeneity (gamma distribution shape parameter = 4.85). Equal weighting and a ts:tv weighting scheme of 1:4 were used in the MP analyses. We conducted a single heuristic search using the ML criteria and 500 bootstrap replicates of heuristic searches using MP criteria. Gaps were treated as missing data and uninformative characters were

excluded from analyses. Because all analyses resulted in similar phylogenetic trees, only the results of the parsimony analysis are presented.

Prezygotic Isolation

We tested for reproductive isolation by combining a male fly from one population with three female flies from another population in replicate cages. We used several, but not all, of the possible pairs of populations in an effort to maximize phylogenetic coverage without unnecessary replication of distant crosses (Table 2). We conducted experimental crosses in four rounds between May, 2000 and March, 2003. In the first round we reciprocally crossed the two populations of *C. whitei.* In the second round, we crossed the Gombak, Soraya, Belalong, and Cameron populations of *C. dalmanni* in every possible combination. In the third round we crossed Bukit Lawang and Bogor with a subset of the other *C. dalmanni* populations. In the final round, we tested the *C. dalmanni* Gombak and *C. whitei* Chiang Mai populations in reciprocal crosses. We also conducted all within-population crosses to estimate within-population fertility, behavior rates, progeny production and copulation success.

To quantify reproductive isolation we scored interest and success in mating using observations and female dissections. We observed flies in transparent Nalgene (Nalge Nunc International, Rochester, NY) mouse cages modified with ventilation and access holes and inverted on pans lined with moist cotton and blotting paper. The day before beginning observations we released three females individually marked with paint into a clean cage. Prior evidence indicates that mating behavior is most frequent at dawn and

dusk (Lorch et al. 1993; Wilkinson et al. 1998a). Therefore, just before lights came on (0900 h) we released one unanesthetized male into each cage. We then timed all successful copulations and tallied all related male behavior not leading to successful mating for the ensuing 2.5 h using a hand-held tape recorder. We define a successful copulation as one that exceeds 30 sec, which is long enough to transfer sperm in *C. whitei* (Lorch et al. 1993). Other behavior was recorded as "pursuit," when a male chased or jumped towards a female but did not land on her, and "copulation attempts," where a male alighted on a female but did not mate or mated for less than 30 sec. Observations on each day were balanced with respect to type of cross with either 15 or 16 cages observed at a time, depending on the round (Table 2).

After observation the flies remained in their cages for one week, after which we dissected females and examined their spermathecae for sperm. We anesthetized a female with carbon dioxide or cold, pulled out the two terminal abdominal segments with spermathecae attached while the female was still alive, and deposited the tissue into a drop of phosphate-buffered saline (PBS, pH = 7.4) on a microscope slide. Spermathecae were then gently squashed under a cover slip and immediately examined for sperm at 400X magnification with dark-field illumination. If any female in a cage contained sperm, we scored the male as successfully transferring sperm. We then calculated the proportion of all replicates of a cross in which sperm was transferred.

Postzygotic Isolation

To quantify postmating and postzygotic isolation, we used the cages described above to score progeny production and hybrid fertility. We collected eggs in food cups for one week after mating observations ended, counted all eclosing offspring by sex and kept hybrids for four weeks to reach reproductive maturity. Then, treating hybrids from reciprocal crosses as different types, we crossed up to five flies of each sex from each hybrid type with each parental population (Table 2). Each hybrid fly was housed with two flies of the opposite sex to ensure a fertile partner. Females were allowed to oviposit in food cups for 10 days, after which we removed and examined their spermathecae for stored sperm. We also counted any offspring by sex that eventually eclosed from the food cups. We scored female hybrids sterile when they produced no offspring but stored motile sperm. We scored male hybrids sterile if no motile sperm were stored by their mates and no progeny were produced. Because we were not able to assess egg fertility rates, we cannot determine whether low progeny counts reflect failure of sperm to fertilize eggs or a failure of embryonic development. Thus, hybrid progeny counts potentially confound postzygotic isolation with postmating prezygotic isolation. Hybrid fertility, however, is purely a measure of postzygotic isolation.

Data Analysis

We first converted observations of pursuit, attempted copulation, and successful copulation into hourly rates. Then we performed linear regression on each of those

measures, as well as the proportion of males that successfully transferred sperm and the number of progeny produced, against genetic distance. Genetic distance was measured as the average pairwise uncorrected proportion of nucleotide substitutions for the 889 bp of mtDNA between individuals from two populations (Figure 2). Our observations are not independent because every data point is a property of two populations and each population is represented in several data points. Thus, we pooled the data from reciprocal crosses and used the regression version of the Mantel test as described in Smouse et al. (1986). We determined statistical significance of our regression coefficients using randomization procedures (Manly 1996), as have other studies of reproductive isolation (Gleason and Ritchie 1998; Moyle et al. 2004; Tilley et al. 1990). We chose to perform 10,000 replicates, which gives the p value a standard error of 0.002 when it is near the alpha level of 0.05; 1000 replicates gives a standard error roughly three times larger. Prior to reporting the results of any parametric analyses, we examined the residuals to ensure compliance with the standard assumptions of normality and homoscedasticity. If an assumption was violated, we transformed the data to correct the problem.

Evidence of asymmetrical reproductive isolation may indicate that pooling data from reciprocal crosses would be inappropriate. We therefore tested each of our 12 pairs of reciprocal crosses for potential asymmetry in copulation, pursuit, and copulation attempt frequency and progeny production using *t*-tests. We also tested for asymmetry in sperm transfer frequency and hybrid fertility using association tests and estimated significance from either a chi-squared statistic or Fisher's exact test. We analyzed the hybrid sexes separately and only performed the analysis if at least six hybrids from each of the two reciprocal crosses had been tested for fertility. To correct for multiple testing,

we applied the sequential Bonferonni correction for type I error within each set of tests (Rice 1989).

To test if hybrid sterility obeys Haldane's rule, we performed a Wilcoxon signed-rank test on the percent fertility of male versus female hybrids. To test if hybrid inviability obeys Haldane's rule we calculated the brood sex ratio (percent male) produced by every between-population cross and performed a Mantel test by regressing sex ratio on genetic distance. Although progeny counts could be influenced by either hybrid inviability or gametic isolation, these data can still be used to test for Haldane's rule for inviability because progeny sex ratio should be independent of sperm-egg incompatibility. We performed two tests to assess the potential influence of meiotic drive on the pattern of progeny sex ratios. In the first we used a regression Mantel test on the percent of drive males, that is, those producing biased sex ratios, against genetic distance. In the second we tested if male drive phenotype was independent of the type of cross (within or between population) using a chi-squared contingency table. We assessed drive phenotype using a chi-squared goodness-of-fit test for departure from a 50:50 sex ratio. Only those males producing at least 20 offspring were tested.

To compare the rate of evolution between different types of reproductive isolation, we first converted all measurements to a common, continuous scale. For premating isolation we divided the mating frequency for each replicate of a between-population cross by the average of all replicates of the two corresponding within-population crosses. Similarly, for postmating isolation we divided the observed progeny count of each between-population replicate by the average progeny count for the maternal population. Then, to compare premating isolation (copulation rate) directly with

postmating isolation (progeny production), we performed another Mantel test. In this test for each cross combination we subtracted scaled progeny production from scaled mating rate, and then regressed the resulting differences against genetic distance. A significant slope would, therefore, indicate that premating isolation evolves either more slowly or more rapidly than postmating isolation. Hybrid sterility could not be analyzed using linear regression methods due to poor fit of the data to a linear model. Instead, we compared male and female hybrid sterility separately to both progeny production and copulation rate using Wilcoxon signed-rank tests. Because the tabular p-values for this test are based on all possible permutations under the binomial distribution, a randomization procedure is unnecessary.

Results

Phylogenetic Relationships

The combined COII and 16S dataset included 889 characters, 184 of which were phylogenetically informative. The weighted analysis (i.e., 1:4 ts:tv) resulted in two most parsimonious trees; a phylogram of the bootstrap consensus tree is presented in Figure 3. Each of the three described species of *Cyrtodiopsis* forms a monophyletic unit, with bootstrap support ranging from 96% for *C. dalmanni* to 100% for the other two species. In addition, all five individuals from each population form a monophyletic unit with significant bootstrap support. The six populations of *C. dalmanni* form two distinct clades which are separated by 5.3% sequence difference.

Prezygotic Isolation

Prezygotic isolation increases monotonically with genetic distance as evidenced by a decline in copulation frequency with increasing population divergence ($P < 0.002$, Figure 4A). Between-population copulation frequencies are always below the within-population averages (Table 3), and the incidence of mating falls nearly to zero at 5% sequence divergence. No pairs of reciprocal crosses exhibited significant asymmetry in copulation frequency (Table 4). The frequency of sperm storage in females (Figure 4B) exhibits a pattern that is similar to copulation frequency in that it declines with increasing genetic distance ($P < 0.002$), but for some between-population crosses sperm storage occurs more often than within populations. This result indicates that sperm were transferred during the week of cohabitation between male and females in at least some cages where no mating was observed during the 2.5 h observation period. Two pairs of reciprocal crosses showed significant asymmetry in this measure (Table 4).

In contrast to the pattern exhibited by copulation and sperm storage, pursuit frequency (Figure 4C) increases with genetic distance ($P = 0.010$), and often exhibits a higher rate of occurrence between than within populations. We failed to detect an effect of genetic distance on copulation attempts ($P = 0.29$, Figure 4D). No significant asymmetry was detected between reciprocal crosses in either of these measures (Table 4).

Postmating Isolation

Progeny counts (Figure 5A) decline with genetic distance ($F = 66.06$, $P < 0.002$), with cross averages falling to zero at 5% sequence divergence. No pairs of reciprocal crosses exhibited significant asymmetry in progeny production (Table 4). Brood sex ratio does not vary with genetic distance ($F = 2.61$, $P = 0.92$), indicating that no sex bias in hybrid viability could be detected. Brood sex ratio does appear to be affected by the presence of males carrying meiotic drive in all populations. Of 166 males that produced enough progeny to be tested for departure from a 1:1 brood sex ratio, 41 (24.7%) had significantly female-biased brood sex ratios. However, the fraction of males expressing female-biased brood sex ratios does not covary with the genetic distance of the cross ($P = 0.155$). Furthermore, the drive phenotype of a male is independent of the type (within or between populations) of cross (chi-square = 0.076, $P = 0.783$).

Hybrid Sterility

Hybrid males were sterile (Figure 5B) except for two crosses involving recently isolated populations. In *C. whitei*, Gombak males crossed with Chiang Mai females (0.83% sequence divergence) produced seven fertile and three sterile males, and the reciprocal cross produced three fertile and seven sterile males. In *C. dalmanni*, Gombak males crossed with Bukit Lawang females (2.2% sequence divergence) produced six fertile and four sterile males. The reciprocal cross between Gombak and Bukit Lawang produced no fertile males, making this the only pair of crosses to show significant

asymmetry (Table 4). In contrast, female hybrid fertility was generally high, averaging 71 ± 11.9% until genetic distance exceeded 5%, above which no fertile hybrids of either sex were found. Hybrid fertility of females is higher than males (Wilcoxon signed-rank test, S = -21, $P = 0.031$), consistent with Haldane's rule for sterility.

Relative Rates of Evolution

We failed to find a difference between the rates at which prezygotic isolation (mating frequency) and postmating isolation evolve (Mantel test, $P = 0.705$). However, male hybrid sterility evolves sooner than both prezygotic isolation (Wilcoxon signed-ranks tests, S = -14, $P = 0.016$) and postmating isolation (S = -14, $P = 0.016$). Female hybrid sterility evolves later than prezygotic isolation (S = 15, $P = 0.0391$), but we failed to detect a difference between female sterility and postmating isolation (S = 5.5, $P = 0.570$).

Discussion

Prezygotic Isolation

Our results indicate that in *Cyrtodiopsis*, as in *Drosophila*, Lepidoptera, and other taxa, prezygotic isolation increases monotonically over time. This pattern is indicative of a gradual accumulation of multiple factors of small effect. Copulation and sperm transfer

frequencies both exhibit a decrease with increasing genetic distance, indicating that as populations become isolated from each other the probability of successful mating decreases. This result indicates that either male or female mating interest is depressed in crosses between genetically distant populations. Because male pursuit behavior increases with genetic distance, it appears that males continue to seek mating opportunities from females that reject them. These results are consistent with mating discrimination evolving more rapidly in females than in males.

The cause of the observed prezygotic isolation is not yet known, because little obvious morphological evolution has taken place between these populations (Swallow et al. 2005). Divergence between populations in traits affecting mate choice appears to be insufficient to explain the observed relationship between mating frequency and genetic distance. Neither body size nor eyestalk allometry differs between populations, but the Belalong population does have a distinctive eyestalk phenotype: instead of protruding horizontally from the head, the eyestalks are at a visibly higher angle. If mating interest was affected by this difference, we would expect to observe asymmetries in behavior in most crosses involving the Belalong population, but this did not occur. Divergence in courtship behavior (Price and Boake 1995) and cuticular hydrocarbons (Coyne and Charlesworth 1997; Coyne et al. 1994) have been shown to produce reproductive isolation in *Drosophila*. However, *C. whitei* and *C. dalmanni* display no obvious courtship behavior (Wilkinson et al. 1998a), that is, there is no ritualized precopulatory touching that might allow flies to sense non-volatile waxes on the cuticle of a potential partner. Whether volatile compounds or other behavioral or morphological cues affect prezygotic isolation in these flies deserves further study.

Postmating and Postzygotic Isolation

We found evidence for Haldane's rule with respect to hybrid sterility but not hybrid inviability. Over the range of genetic distances we sampled, progeny sex ratio did not change as a function of genetic distance, as would be expected if male hybrids were less viable than female hybrids. Also, the frequency of males expressing meiotic drive was consistent with previously published estimates for *Cyrtodiopsis* (Wilkinson et al. 2003). In contrast, sex bias in hybrid sterility was apparent even for populations with very little sequence divergence, before complete sterility became the rule. The lowest sequence divergence we sampled at which all female hybrids were sterile was 5.4%, whereas the lowest value at which all male hybrids were sterile was 2.3%, a 2.4-fold difference. Thus, hybrid male sterility has evolved considerably faster than hybrid female sterility in these animals.

That we observed Haldane's rule for hybrid sterility and not inviability may not be surprising. Although both types of postzygotic isolation are thought to be caused by the accumulation of deleterious epistatic (Dobzhansky-Muller) incompatibilities (Dobzhansky 1937b; Muller 1940; 1942; Orr and Turelli 2001), separate genetic causes may be involved (Presgraves and Orr 1998). Sterility-causing incompatibilities accumulate more rapidly in male than female *Drosophila* (Wu and Davis 1993), and by extension other male-heterogametic taxa, possibly due to sexual selection (Wu et al. 1996). Inviability-causing incompatibilities become visible first in the heterogametic sex due to the expression of deleterious X-linked recessive mutations when in the hemizygous state (Orr 1993; Turelli and Orr 1995). If one mechanism operates faster than

the other, it should be possible to detect species pairs that diverged in the time period between the appearance of Haldane's rule for sterility and inviability. Some of the *Cyrtodiopsis* populations examined in this study are consistent with this prediction. However, even though we found no change in the brood sex ratio of hybrids with genetic distance, we did find a highly significant decline in progeny production. As Haldane's rule is an early and nearly ubiquitous form of postzygotic isolation (Coyne and Orr 1989a), this decline may reflect gametic isolation instead of or in addition to hybrid inviability. Further study to determine the fertilization rate of eggs in between-population crosses is needed to separate hybrid inviability from gametic isolation.

Relative Rates of Evolution

We found two differences between hybrid sterility and postmating isolation in *Cyrtodiopsis*. First, hybrid male sterility, but not hybrid female sterility, has evolved faster than postmating isolation. Even if we have completely confounded sperm-egg incompatibility with hybrid inviability, our measure of postmating isolation sets an upper limit on the amount of hybrid inviability. Thus, we can be certain that male hybrid sterility evolves faster than hybrid inviability. This result shows the advantage of analyzing forms of reproductive isolation separately rather than combining traits into a composite isolation index. Second, hybrid sterility in *Cyrtodiopsis* shows a different pattern of evolution from postmating isolation. Postmating isolation increases gradually with no detectable change in brood sex ratio over the range of sequence divergence in this study. Sterility, however, evolves abruptly in a sex-specific pattern. Male hybrid

sterility is incomplete at low genetic distance, but quickly becomes complete. Female hybrids all have similar fertility at genetic distances below 5% but are sterile above that level.

Whether these results differ from other taxa is difficult to determine because other studies have either lacked hybrid sterility information (Mendelson 2003), combined sterility and inviability data into one isolation index to compare prezygotic to postzygotic effects (Coyne and Orr 1989a; Sasa et al. 1998; Zouros 1973), or lacked prezygotic isolation data (Presgraves 2002; Price and Bouvier 2002; Sasa et al. 1998). However, a genetic study of postzygotic isolation in *Drosophila* that tested many small introgressions found more male hybrid sterility loci than either hybrid inviability or female hybrid sterility loci (Tao and Hartl 2003). Moreover, hybrid sterility evolves faster than inviability in many taxa (Presgraves 2002; Price and Bouvier 2002; Sasa et al. 1998; Wu 1992). These studies also showed compliance with Haldane's rule, indicating faster evolution of sterility in the heterogametic hybrid sex. Thus, other taxa seem to follow the same pattern of evolution seen in *Cyrtodiopsis*: first male hybrid sterility, then female hybrid sterility, then hybrid inviability. How the rates of evolution of male and female hybrid sterility and inviability considered separately compare to prezygotic isolation in these other taxa is not known. In addition, quantitative measurement of postzygotic isolation will be necessary to determine if hybrid sterility also evolves abruptly in those taxa.

Accelerated Evolution of Male Hybrid Sterility

Postzygotic isolation could evolve rapidly by more than one mechanism. Possibilities include endosymbionts such as *Wolbachia* (Breeuwer and Werren 1990; Werren et al. 1986), intragenomic conflict from selfish genetic elements (Frank 1991; Hurst and Pomiankowski 1991), antagonistic coevolution (Rice 1996; Rice 1998), and sexual selection (Wu et al. 1996; Wu and Davis 1993). However, not all mechanisms will produce accelerated male hybrid sterility in relation to female hybrid sterility, hybrid inviability, and prezygotic isolation. Tao and Hartl (2003) argued that experimental data supports a new faster-heterogametic-sex hypothesis of Haldane's rule. This hypothesis, which is related to an earlier hypothesis that divergent sex chromosome meiotic drive systems contribute to Haldane's rule (Frank 1991; Hurst and Pomiankowski 1991), suggests a major role of genomic conflict in the rapid evolution of male hybrid sterility. Intergenomic and cytoplasmic-nuclear genome conflicts affecting progeny sex ratio may be particularly important. At least two types of conflict, meiotic drive and sperm competition, are present and affect one another in *C. whitei* (Fry and Wilkinson 2004; Wilkinson and Fry 2001) and are intimately linked to sexual selection (Wilkinson et al. 1998b; Wilkinson et al. 2003). Future research is needed to determine how much influence these forms of conflict and sexual selection have on speciation in this genus.

Table 2: List of crosses, sample sizes and proportion nucleotide substitutions. Observation sample sizes before the slash are for the cross as listed in the left-hand column (male x female); those after the slash are for the reciprocal cross. For number of hybrids tested, the number before the slash represents flies whose male parent is listed first in the cross column; those after the slash are for flies whose female parent is listed first.

Cross	Observation sample size	Genetic distance	Number of hybrids tested	
			Male	Female
Within *C. dalmanni*				
Gombak x Soraya	16/15	0.0207	10/3	10/10
Gombak x Cameron	15/16	0.0576	0/0	0/1
Gombak x Belalong	14/15	0.0596	1/0	1/0
Gombak x Bukit Lawang	16/15	0.0211	10/10	10/10
Soraya x Cameron	15/15	0.0534	0/0	0/0
Soraya x Belalong	15/15	0.0533	0/0	2/0
Soraya x Bukit Lawang	16/16	0.0035	4/10	9/10
Cameron x Belalong	14/16	0.0399	3/0	10/4
Cameron x Bogor	15/15	0.0337	0/0	0/0
Belalong x Bogor	15/15	0.0376	10/10	10/10
Within *C. whitei*				
Gombak x Chiang Mai	15/17	0.0083	10/10	10/10
Between *C. dalmanni* and *C. white*				
Gombak x Chiang Mai	16/16	0.0783	0/0	0/0

Table 3: Within-population average (± 1 SE) for hourly behavior frequencies, sperm transfer rate, and progeny production over a one-week period

Population	n	Copulation	Sperm transfer	Pursuit	Copulation attempts	Progeny count
C. dalmanni						
Bukit Lawang	15	1.9 ± 0.61	1.000	10 ± 4.1	0.07 ± 0.067	36 ± 8.6
Bogor	15	5.0 ± 0.42	1.000	6 ± 1.3	1.3 ± 0.34	130 ± 14
Belalong	14	2.8 ± 0.64	0.929	9 ± 1.4	0.5 ± 0.20	28 ± 8.3
Cameron	15	1.8 ± 0.26	0.867	5.4 ± 0.63	0.13 ± 0.091	24 ± 6.0
Gombak	15	4.6 ± 0.76	0.933	4 ± 1.1	0.13 ± 0.091	60 ± 12
Soraya	15	3.6 ± 0.38	1.000	4 ± 1.0	0.13 ± 0.091	70 ± 13
C. whitei						
Gombak	15	2.3 ± 0.55	1.000	4 ± 1.8	0.07 ± 0.067	38 ± 7.5
Chiang Mai	17	5.2 ± 0.47	1.000	4.8 ± 0.83	0.4 ± 0.24	65 ± 8.1

Table 4: Results of asymmetry tests. Table values are *t* (first four columns) or chi-square (last two columns) statistics. Numbers before the slash in the last column are for male hybrid fertility, after are for female hybrid fertility. Significance was calculated using a sequential Bonferroni correction applied to each column of *P*-values (Rice 1989).

Cross	Progeny count	Copulation frequency	Pursuit frequency	Attempt frequency	Sperm transfer	Hybrid fertility
Bukit Lawang x *C.d.* Gombak	1.91	-0.91	-1.46	-1.59	[2]	10.77*/1.05
Bukit Lawang x Soraya	0.18	3.05	-2.17	1.25	5.24	[2]/1.72
Bogor x Belalong	2.71	3.06	1.84	-0.49	5.00	[2]/1.05
Bogor x Cameron	1.00	-0.87	0.79	-1.05	0.37	[3]
Belalong x Cameron	-3.11	-3.24	-2.34	1.47	16.63***	[3]
Belalong x *C.d.* Gombak	-1.51	-1.00	-1.35	-0.65	0.58	[3]
Belalong x Soraya	-1.46	[1]	-1.07	[1]	7.50	[3]
Cameron x *C.d.* Gombak	1.00	1.00	-0.28	0.15	4.21	[3]
Cameron x Soraya	[1]	1.86	1.17	-0.59	[2]	[3]
C.d. Gombak x Soraya	0.11	-0.08	0.70	-0.58	[2]	[3]/1.17

Chiang Mai x *C.w.* Gombak	-0.71	1.87	1.39	-0.72	[2]	3.20/[2]
Chiang Mai x *C.d.* Gombak	[1]	1.89	1.46	-2.20	16.76***	[3]

***$P < 0.001$; **$P < 0.01$; *$P < 0.05$

[1] Statistic < 0.001

[2] Test not performed because one column of the contingency table contained only sperm transfer or hybrid fertility rates of zero

[3] Test not performed because there were not enough surviving hybrid offspring

Figure 2: Stalk-eyed fly collection localities in the Sunda shelf region of Southeast Asia. For site details and species collected, see text.

Figure 3: Maximum-parsimony hypothesis for the phylogenetic relationships among populations using 889 bp of mitochondrial DNA (cytochrome oxidase II and 16S ribosomal RNA) from five flies per population. Numbers on each branch indicate bootstrap support.

Figure 4: Prezygotic isolation measures are plotted against genetic distance (p, the proportion uncorrected DNA sequence changes). Open circles show the within-population values, filled circles show comparisons between conspecific populations, and the filled square represents the single between-species comparison. Within-population values indicate the average percent sequence divergence among five flies from each population. (A) Number of copulations observed per hour in cages containing one male and three females ($R^2 = 0.74$, b = -22.21, 95% confidence interval = -28.54, -15.86). (B) Proportion of replicate cages in which at least one female contained sperm in her spermathecae after one week ($R^2 = 0.68$, b = -11.63, 95% CI = -15.45, -7.85). (C) Number of times males chased females per hour ($R^2 = 0.20$, b = 30.10, 95% CI = 3.55, 46.00). (D) Number of unsuccessful copulation attempts per hour ($R^2 = 0.01$).

Figure 5: Postzygotic isolation measures are plotted against genetic divergence (p, the proportion uncorrected mtDNA sequence changes). (A) Progeny production, after $\log_{10}$ transformation, with symbols as in Figure 4 ($R^2 = 0.74$, b = -57.61, 95% CI -73.95, -

41.24). (B) Proportion of hybrids that produced progeny when mated. Open circles represent within-population males, closed circles represent male hybrids. Squares represent female hybrids. Within-population female values could not be calculated from these data.

Fig. 2


Chiang Mai
800 km
Bt. Lawang
Cameron
Belalong
Gombak
Soraya
Bogor

Fig. 3

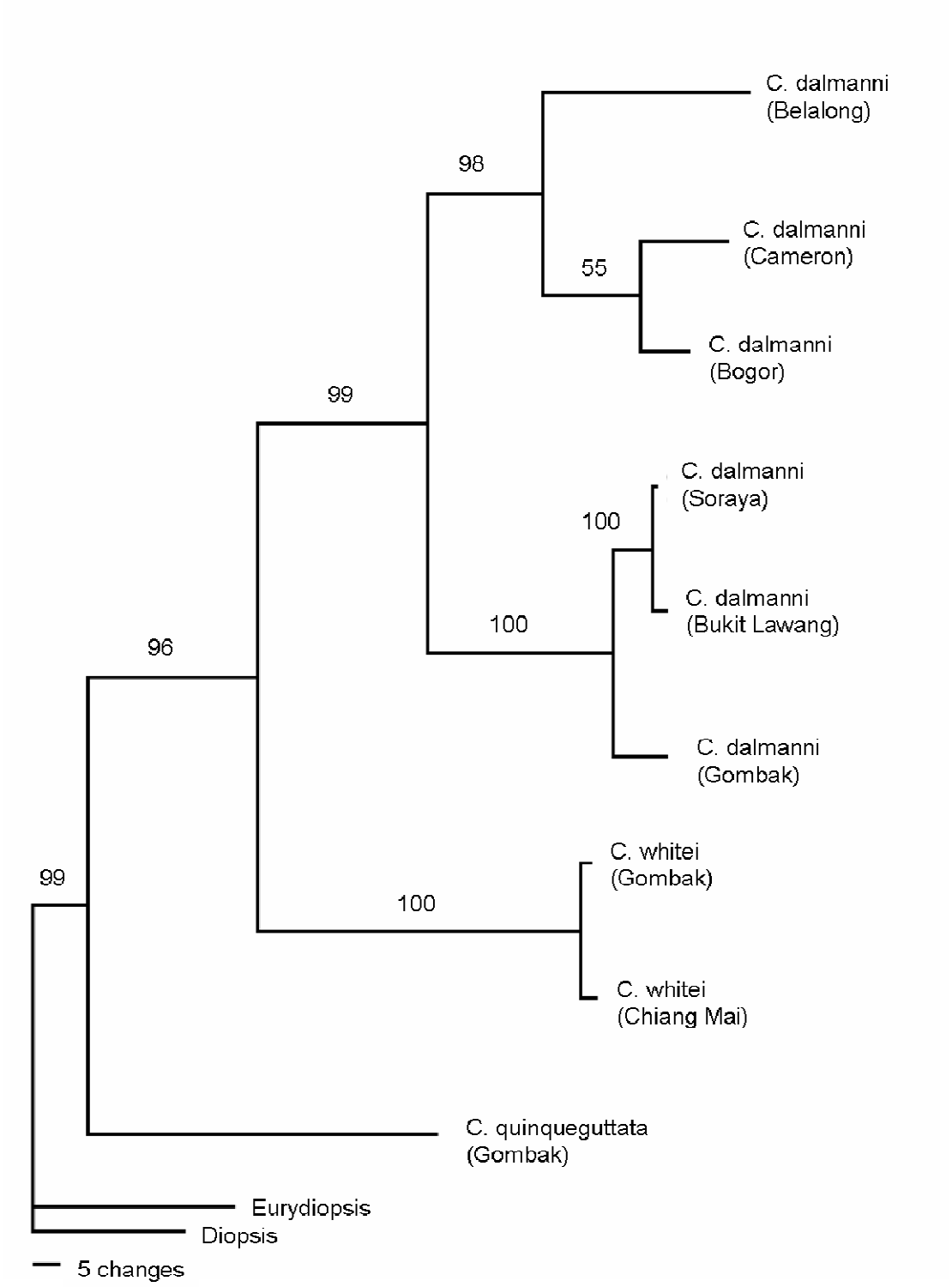

C. dalmanni
(Belalong)
98
C. dalmanni
(Cameron)
55
C. dalmanni
(Bogor)
99
C. dalmanni
(Soraya)
100
C. dalmanni
(Bukit Lawang)
100
96
C. dalmanni
(Gombak)
C. whitei
(Gombak)
99
100
C. whitei
(Chiang Mai)
C. quinqueguttata
(Gombak)
Eurydiopsis
Diopsis
5 changes

Fig. 4

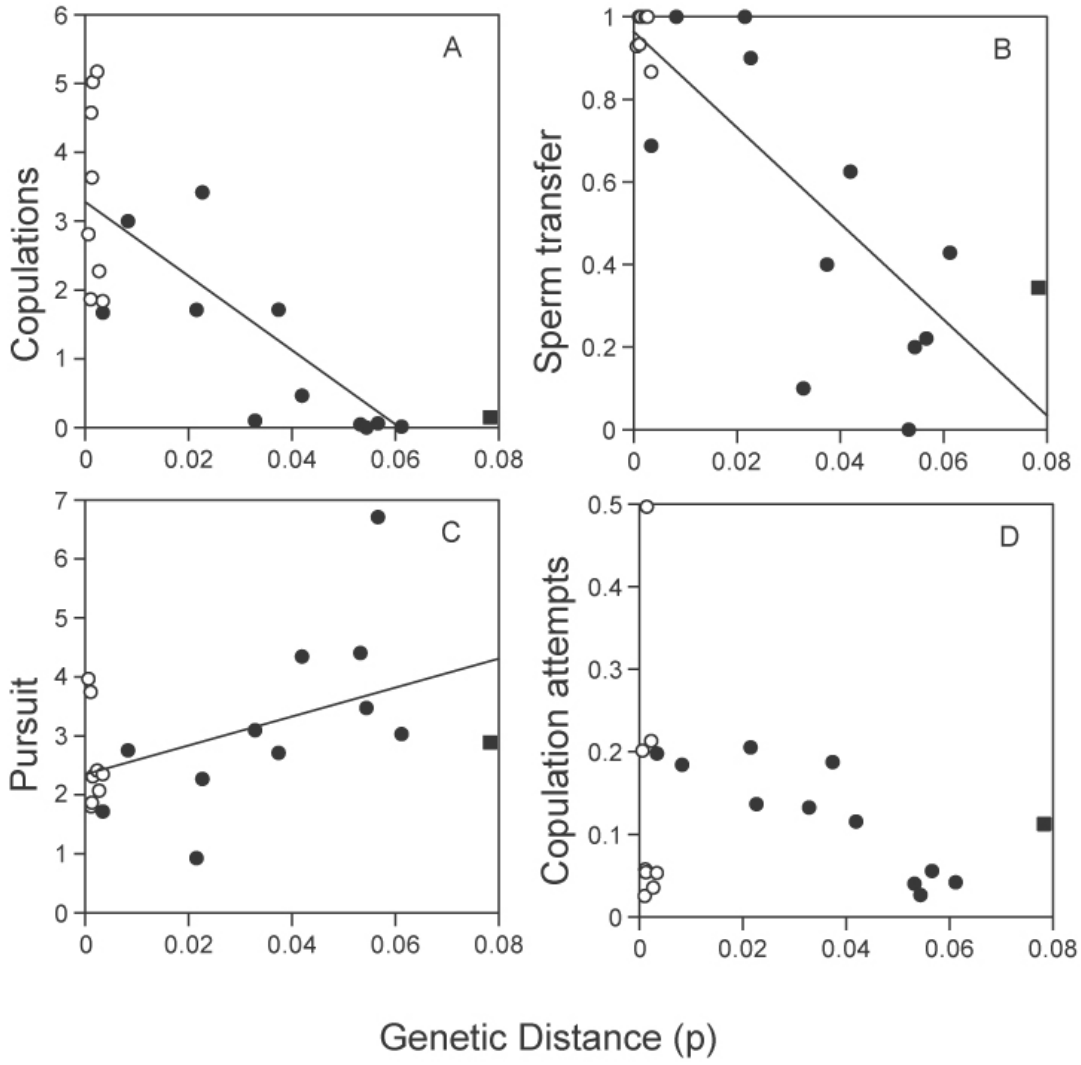
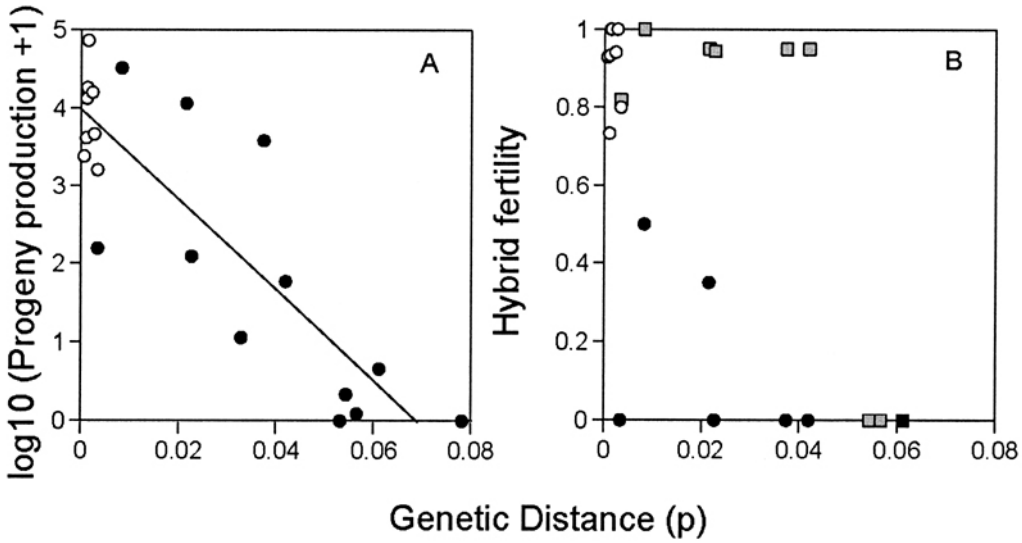

A
B
C
D
Copulations
Sperm transfer
Pursuit
Copulation attempts
Genetic Distance (p)
0
0.02
0.04
0.06
0.08

Fig. 5


log10 (Progeny production +1)
A
Hybrid fertility
B
Genetic Distance (p)
0
0.02
0.04
0.06
0.08
0.2
0.4
0.6
0.8
1
2
3
4
5

Chapter 2: Reduced polymorphism and increased divergence associated with meiotic drive in the stalk-eyed fly *Cyrtodiopsis dalmanni*

Abstract

The meiotic drive hypothesis of Haldane's rule states that divergence between incipient species at loci involved in sex chromosome meiotic drive leads to early hybrid incompatibilities. I sequenced autosomal and drive-associated X-linked loci in the stalk-eyed fly *Cyrtodiopsis dalmanni* and analyzed the sequences for patterns of polymorphism and divergence. I show that there is greatly reduced polymorphism associated with meiotic drive, which is consistent with the action of positive selection and hitchhiking of linked genes. I also show that the X^D and X^{ST} chromosomes have diverged significantly with respect to one another, and to a greater extent than the autosomal loci. These results are consistent with antagonistic coevolution within the complex of meiotic drive-associated loci, as expected if drive is causing Haldane's rule.

Introduction

Hybrid dysfunction in the heterogametic sex, a phenomenon known as Haldane's rule (Haldane 1922), is an important and complex milestone on the way to the complete isolation of new species (Coyne and Orr 2004; Orr 1997). At least three hypotheses describe the processes generally believed to contribute to Haldane's rule (Coyne and Orr 2004; Presgraves and Orr 1998). First, the dominance theory (Muller 1940; Muller 1942; Orr 1993; Turelli and Orr 1995) states that if the epistatic interactions leading to hybrid dysfunction are, on average, at least partially recessive, loci on a hemizygous sex chromosome will contribute more to sterility and inviability than those on an autosome. An autosomal allele, because it may be paired with another allele producing the dominant phenotype, is less likely to lead to the expression of a recessive hybrid dysfunction. Second, the faster-male theory (Wu et al. 1996) states that, because there is intense competition between males to achieve fertilizations, male reproductive genes evolve faster than female reproductive genes and lead to earlier male than female hybrid sterility. This explanation applies specifically to the evolution of sterility in male-heterogametic species. The third hypothesis is called the faster-X theory (Charlesworth et al. 1987). According to this hypothesis, which is regarded as less important than the other two (Orr 1997), higher substitution rates of favorable, partially recessive mutations on a hemizygous chromosome lead to faster evolution on the X than on autosomes.

A fourth, more controversial, explanation for Haldane's rule is that divergence at loci affecting sex chromosome meiotic drive may lead to hybrid male sterility and possibly inviability in male-heterogametic species (Frank 1991; Hurst and Pomiankowski

1991). While some evidence supports this meiotic drive hypothesis (Dermitzakis et al. 2000; Orr and Irving 2005; Tao et al. 2001), many questions must be answered in order to determine whether meiotic drive is a major factor in the evolution of hybrid dysfunction. One such question is whether the evolutionary change occurring at drive-associated loci is capable of leading to early hybrid incompatibilities. Genes that evolve rapidly under the influence of selection may be more likely to lead to initial hybrid incompatibilities than genes which evolve more slowly. One approach to determine whether meiotic drive is evolving under the influence of selection is to examine DNA sequence variability for characteristic changes known to be produced by selection.

Sex chromosome meiotic drive is a selfish genetic system which is characterized by non-Mendelian transmission of sex chromosomes (Sandler and Novitski 1957); most typically, one observes transmission of the X at the expense of the Y (e.g., Carvalho et al. 1989; Gershenson 1928; James and Jaenike 1990; Presgraves et al. 1997; Stalker 1961; Sturtevant and Dobzhansky 1936). A driving X chromosome (X^D) outcompetes the non-driving X chromosome (X^{ST}) and, if unimpeded by selection, is predicted to proceed to fixation (Hamilton 1967). However, multiple levels of selection act on the X^D chromosome and the flies that carry them. When drive is active in a population, females mating with non-driving males have higher fitness because sons are valuable in a female-biased population (Capillon and Atlan 1999; Curtsinger and Feldman 1980; Jaenike 1996). Traits indicative of a male's drive status may then evolve, allowing females to choose mates that will produce a favorable offspring sex ratio (Lande and Wilkinson 1999; Wilkinson et al. 1998b). Selection to avoid producing the more-abundant sex favors the spread of autosomal drive suppressors, which act to correct the imbalance in

the sex ratio (Carvalho and Klaczko 1993; Hauschteck-Jungen 1990). The Y chromosome faces selection to avoid exclusion from sperm, which favors the evolution of Y-linked suppressors producing drive resistance (Carvalho et al. 1997; Hauschteck-Jungen 1990; Mercot et al. 1995; Stalker 1961). The driving X also faces selection to increase its own success, accumulating X-linked modifiers of the original drive locus (Montchamp-Moreau and Cazemajor 2002). And finally, decreased female fecundity, male fertility, and viability have all been reported in conjunction with meiotic drive in flies (Beckenbach 1996; Curtsinger and Feldman 1980; Edwards 1961; Wilkinson et al. 2006), and likely result in selection against the drive chromosome.

Most of the types of selection listed above are forms of positive selection, in which an allele that confers a benefit to itself (in the case of the drive allele or an X-linked modifier), its chromosome (a Y-linked suppressor), or the organism (any suppressor) increases in frequency. Fecundity, fertility, or viability costs, however, may cause background selection, which decreases the frequency of a deleterious allele. Heterozygous advantage in females may produce balancing selection, which leads to an intermediate allele frequency. The complex cycle of evolution of drive and its suppressors and modifiers can lead to a polymorphism of drive and non-drive phenotypes (Carvalho et al. 1997; Jaenike 1996) or to complete masking of the drive phenotype (Cazemajor et al. 1997; Dermitzakis et al. 2000; Orr and Irving 2005). These different forms of selection can also lead to different patterns of polymorphism at the DNA sequence level. Under positive selection a successful allele increases in frequency, which means other alleles at the same locus must necessarily decrease in frequency. The resulting change in the frequency spectrum affects both the locus under selection and

others linked to it, due to a process known as "genetic hitchhiking" (Kaplan et al. 1989; Kojima and Schaffer 1967; Maynard Smith and Haigh 1974). When hitchhiking occurs the remaining allelic diversity tends to be concentrated in rare alleles with a marked deficit of intermediate-frequency alleles (Aguade et al. 1989; Braverman et al. 1995). DNA polymorphism is also reduced under background selection (Charlesworth et al. 1993b), but the resulting allele frequency distribution is much less severely skewed than under positive selection (Charlesworth 1996; Charlesworth et al. 1995). In contrast, balancing selection favors polymorphism and tends to leave an allele frequency distribution higher in the center and deficient in the tails when compared to the neutral expectation (Fay and Wu 2000).

The prevailing opinion is that in *Drosophila*, while some contribution has come from demographic processes (Hutter et al. 2007) and background selection (Jensen et al. 2002), repeated selective sweeps are responsible for most observed cases of reduced DNA polymorphism (Andolfatto and Przeworski 2001; Begun and Aquadro 1992; Glinka et al. 2003; Quesada et al. 2003). In particular, several species in the genus show low sequence polymorphism in chromosomal regions with reduced recombination (Aguade et al. 1994; Aguade et al. 1989; Jensen et al. 2002; Martin-Campos et al. 1992; Miyashita 1990; Stephan and Langley 1989; Stephan and Mitchell 1992). Genetic hitchhiking reduces DNA polymorphism more effectively in regions of low recombination (Kaplan et al. 1989). In addition, simulations suggest that when recombination is extremely low, reduced polymorphism is most likely due to hitchhiking, not background selection, and polymorphism is most effectively reduced when positive selection acts alone rather than in combination with background selection (Kim and Stephan 2000).

Reed et al. (2005) point out that in *Drosophila*, the selection coefficients attributable to drive appear to be strong, and speculate it is not a coincidence that drive is found in areas of the X chromosome known to have both low recombination rates and low polymorphism. One study (Derome et al. 2004) also found evidence of a recent, meiotic drive-associated selective sweep that severely reduced polymorphism in one area of the *D. simulans* X chromosome. An interesting contrast to Derome et al. (2004) is a study conducted on meiotic drive in *D. recens* by Dyer et al. (2007). Very little sequence polymorphism was found on the *D. recens* drive X chromosome, but analysis suggested that the chromosome is no longer experiencing selective sweeps, and is instead accumulating deleterious alleles and may be on its way to being lost. The *D. recens* study illustrates that while repeated selective sweeps may leave an impact on patterns of DNA polymorphism, the cycle need not continue indefinitely.

Like most meiotic drive systems in *Drosophila*, the one studied by Derome et al. (2004) includes highly effective suppressors, and it is rare to see a wild population with a significant proportion of males harboring unsuppressed meiotic drive (Jaenike 2001; James and Jaenike 1990). Suppressors will increase in frequency due to positive selection and contribute to an overall pattern of reduced DNA polymorphism. A drive polymorphism without active suppression is likely maintained through background or balancing selection that counteracts the transmission advantage of the X^D chromosome. In such a polymorphism, the pattern of variation in DNA sequence should reflect a contribution of negative or balancing selection as well as positive selection.

The stalk-eyed fly *Cyrtodiopsis dalmanni* provides an intriguing system for studying X chromosome meiotic drive. Multiple populations in Southeast Asia possess

high frequencies of X chromosome drive, have close phylogenetic relationships (Wilkinson et al. 2003), and exhibit different degrees of reproductive isolation (Christianson et al. 2005). One or more inversions on the X chromosome are associated with meiotic drive, and there is little evidence of recombination occurring between X^D and X^{ST} chromosomes (Johns et al. 2005). In the wild, drive persists in a natural polymorphism of X^D and X^{ST} chromosomes, with female-biased population sex ratios (Wilkinson and Reillo 1994) and between 8% and 25% of males producing biased sex ratios (Wilkinson et al. 2003). The high frequency of drive in the wild suggests that unlike in *D. recens*, meiotic drive in *C. dalmanni* is not nearing extinction. Published (Johns et al. 2005) and unpublished work (Chapter three of this dissertation) suggests that variation in drive is caused by Y-linked and autosomal suppressors and additional cryptic (completely suppressed) drivers, and earlier studies report the presence of Y-linked modifiers (Presgraves et al. 1997; Wilkinson et al. 1998b). The combined presence of X chromosome drive, suppressors, and cryptic drive suggests a history of repeated selective sweeps. However, the apparent natural drive polymorphism is consistent with ongoing balancing selection (Wilkinson et al. 2006).

Here I use two populations of *C. dalmanni* to conduct a study on the effects of X chromosome drive on patterns of DNA sequence evolution. I sequence two X-linked loci at opposite ends of a large, drive-associated inversion, and two autosomal loci which consist of one coding region and one untranscribed region of the same gene. I examine the sequences for patterns of polymorphism and divergence and perform analyses to test for departure from neutral expectations. I then consider the results in light of the predicted patterns left behind by selective sweep, background and balancing selection

models. I then discuss the implications of these results on the meiotic drive hypothesis of Haldane's rule.

Materials and Methods

DNA Samples

Thirteen male *Cyrtodiopsis dalmanni* were captured in 1999 near the Soraya research station in Aceh province, Indonesia. Five of those males were found to produce offspring in a sex ratio not significantly different from 50:50 (i.e. were presumed to carry X^{ST}, and were non-drivers), but the remainder were not tested before they died. All 13 were preserved in 70% ethanol, and their DNA extracted using DNEasy extraction kits (Qiagen). Twenty-five other males were included in this study, all from the laboratory population originally collected in 1999 near Ulu Gombak, in peninsular Malaysia. That population has since been continuously maintained, at about four generations per year, with at least 100 breeding females. In 2004, a screening project was undertaken to identify males from the Gombak population that carry sex chromosome meiotic drive (Wilkinson et al. 2006). Out of 81 males tested, 15 driver males (i.e. those producing significantly female-biased offspring sex ratios) were found. 11 drive and 14 non-driving males from that study are used here. DNA had been previously extracted from those 25 flies using the DNEasy kit and stored at -80°C.

DNA Sequence Generation and Preparation

Four DNA segments, two autosomal and two X-linked, were sequenced for this study. The autosomal loci included one coding segment and one untranscribed region (UTR) from the same gene, which totaled 921 bp in the alignment used for analysis (see Table 5 for details and primer sequences). This locus is putatively identified as *bangles and beads* (*bnb)*, on the basis of a probability of $7*10^{-36}$ (R. Baker, pers. comm.) for the best match returned from a BLAST search against *Drosophila melanogaster* genome sequence. In *D. melanogaster bnb* is located on the X chromosome, but in *C. dalmanni* it maps to one of the two autosomes (S. Christianson, unpublished data). I also sequenced two regions of the X chromosome, approximately 40 cM apart. One region included part of a gene, *cryptocephal (crc),* that contains a tandemly repeated amino acid (glutamine) while the other region contains a dinucleotide microsatellite (ms125, Wright et al. 2004) and is known to segregate with X chromosome meiotic drive (Johns et al. 2005). In the final alignment the two X-linked loci totaled 1172 bp.

Primers for sequencing came from several sources. Primers for *bnb* coding and UTR sequence came from an expressed sequence tag (EST) project on *Cyrtodiopsis* eye-antennal imaginal discs (Baker and Wilkinson, unpublished data). I then designed additional primers to target sequence fragments less than 800 kb in length. For *crc* I used primers designed for *Cyrtodiopsis* by Dr. Richard Baker (unpublished). For *ms125* I used one primer previously designed for microsatellite genotyping purposes (Wright et al. 2004) and personally designed others to obtain a larger fragment for this sequencing study. Dr. Richard Baker provided sequence from a fosmid clone that was identified as

carrying *ms125* (unpublished data). In several cases more than two primers are listed for the loci in Table 5. For the autosomal loci, this reflects heterozygosity for indels near a primer site. Because such indels make sequence interpretation difficult, I designed additional primers to fall inside the location of the indel and repeated the sequencing process for affected samples. For *crc*, the two external primers bracket a fragment that was too long to sequence reliably, so the internal primers allow the locus to be processed in two smaller, overlapping fragments.

These four DNA segments were first amplified using PCR. Each 25 μL reaction included 2.5 μL 10x Invitrogen PCR buffer, 250 μM dNTPs, between 1.5 and 3.0 mM $MgCl_2$ (optimized for each primer pair), 625 μM of each primer, and 0.625 units of recombinant *Taq* polymerase (Invitrogen, Foster City, CA). The annealing temperature was optimized for each primer pair (see Table 5 for primer sequences). Following an initial denaturation step of two minutes at 94°, the amplification conditions consisted of 35 cycles with 30 s at 94°, 30 s at the annealing temperature and 45 s at 72°. A small amount of each completed PCR sample was run on an agarose gel with a Low Mass DNA Ladder (Invitrogen) to quantify the amount of DNA fragment in the product. I then cleaned the PCR products using 2.5 units of Exonuclease I (USB, Cleveland, OH), 0.25 units of shrimp alkaline phosphatase (USB) and 0.5 μL of SAP dilution buffer (USB) per each 5 μL of PCR product. The mixture was put on a thermal cycler for 30 min. at 37° and 15 min. at 80°. Cycle sequencing was performed in both directions on each cleaned PCR product using ABI Big Dye v3.1 kits. Each 10 μL reaction contained 1 μL Big Dye Ready Reaction Mix, 1.5 μL Big Dye buffer, 3.3 pmol primer, and approximately 20 ng template. Thermal cycling conditions followed the manufacturer's recommendations.

Sequencing reaction products were cleaned by isopropanol precipitation, resuspended in HiDi formamide (ABI), and run on either an ABI 3100 or 3730 automated genetic analyzer.

Each sequence file was checked and edited using Chromas Lite (version 2.01; Technelysium Pty Ltd), and then the sequences were manually aligned using BioEdit (v7.0.5.3, Hall 1999). Because I did not clone individual strands of DNA prior to sequencing, heterozygous individuals could be identified by the presence of two nucleotides at one base position in the sequence data files. In the case of individuals with multiple heterozygous bases, I could not separate the heterozygous sequences into true haplotypes. Instead I described the diploid sequence from each autosomal locus using two "pseudo-haplotypes" per individual. The two pseudo-haplotypes were identical at all homozygous base positions, but were each assigned one of the two nucleotides at heterozygous positions. This allowed me to accurately count alleles at each variable position and assess polymorphism within populations and divergence between them. Because diopsid males have one X chromosome and two of each autosome (Wolfenbarger and Wilkinson 2001), each alignment contained one sequence per individual in the case of an X-linked locus and two in the case of an autosomal locus. I removed all base positions associated with repetitive DNA, indels and missing data before analyzing the aligned sequence, but used the information in the repetitive regions in separate analyses (see below).

Molecular Genetic Analysis

I used DnaSP v4.10.9 (Rozas et al. 2003) to calculate several measures of polymorphism and divergence, including the number of polymorphic sites, number of haplotypes, haplotype diversity (H), π, and θ. For each chromosome and each locus within a chromosome I then calculated two D statistics. Tajima's D (Tajima 1989) detects departure from neutrality, which is useful for two reasons. First, it can help establish whether *bnb* is experiencing strong selection that would make it inappropriate to use for comparison to the X chromosome, and second, it can reveal whether the X^{ST} chromosome is evolving more like X^{D} or an autosome. I also estimated Fu and Li's D* (Fu and Li 1993) to detect and differentiate between balancing and purifying selection. I calculated the D and D* statistics separately for the Gombak X^{D} and X^{ST} samples and again for all pooled Gombak samples.

I used DnaSP to perform two between-population analyses: an HKA test and a set of K^*_{ST} tests. The HKA test (Hudson et al. 1987) detects selection at a locus by comparing polymorphism and divergence using sequence from at least two loci and two populations. I used this test to look for evidence of selection on the X^{D} chromosome by comparing it to the autosomal sequence using males carrying the Gombak X^{ST} as an outgroup. I performed the same comparison between all Gombak X chromosome sequences and the Soraya X sequences. I performed K^*_{ST} tests (Hudson et al. 1992) to examine genetic divergence between the driving- and non-driving-male Gombak samples, and between the Gombak and Soraya population samples. *P* values for these tests were calculated using permutations with 1000 replicates.

It should be noted that three Soraya X chromosomes were quite similar to one another but quite different from the others, as is illustrated in the *crc* segment of Figure 6. Because these three sequences are similar to one another, the *crc* alignment does not include many singleton polymorphisms. Of these three flies, however, only one yielded sequence for locus *ms125* after repeated attempts. Including that one sequence in the analysis of *ms125* or the pooled X chromosome sequence led to large numbers of singleton polymorphisms, which disproportionately affected some of the statistics. For that reason it was excluded from the above analyses of *ms125* and the pooled X chromosome sequence.

While repetitive DNA regions were removed from sequence alignments prior to performing the above analyses, they also contain information on polymorphism and divergence that reflects a shorter time period due to their relatively high mutation rates. The sequenced region for locus *ms125* contained four different repetitive regions – the expected dinucleotide microsatellite repeat, but also a polythymine repeat, a six-bp repeat, and an eight-bp repeat. The *crc* sequence contained a polyglutamine (CAA or CAG) repeat. To assess variation at these repeat regions I counted the number of repeats in each of the five regions and then calculated allelic diversity using the following equation:

$$H = \frac{n}{n-1}\left(1 - \sum_{i=1}^{k} p_i^2\right)$$

(Nei 1987). This is the same equation used by DnaSP to calculate haplotype diversity of DNA sequence. In this equation, H is allele (or haplotype) diversity, n is the number of

chromosomes sampled, p_i is the frequency of the *i*th allele (which in this case is the number of repeats present at one of the five regions), and *k* is the number of alleles.

Phylogenetic Analysis

To determine the evolutionary history of the alleles at each of the loci in this study, I used PAUP* v.4 (Sinauer, Sunderland, MA) to construct separate neighbor-joining (NJ) trees of the autosomal and X-linked sequence. Unlike DnaSP, PAUP* does not exclude nucleotide positions with missing data. Therefore, I was able to include the ends of the alignments which were trimmed before analysis in DnaSP and which contained additional polymorphic sites. I again excluded repetitive sequences and indels where the alignment could not be unambiguously determined. The trees were constructed using the parsimony criterion and rooted by using the Soraya population as an outgroup to the Gombak population (cf. Wilkinson et al. 2003).

Results

The most striking result is the complete lack of polymorphism on the X^D chromosomes, and the difference between the X^D chromosome sequences and all other sequences. All diversity measures for Gombak X^D sequences equaled zero (except for the number of haplotypes, which was one; see Tables 6 and 7). Figure 6 shows the DNA sequence polymorphism, illustrating the lack of variability in Gombak X^D sequences but similar levels of variability in autosomes from the two groups of Gombak samples and in

the Gombak X^{ST} and Soraya X chromosomes. The sequence haplotype diversity of the Soraya X chromosome equaled 1.00, for the Gombak X^{ST} chromosome equaled 0.89, and for the Gombak X^{D} chromosome equaled 0.00. An ANOVA of X chromosome haplotype diversity by population shows that this difference is highly significant ($F = 683.88$; $P < 0.0001$). Figure 7 summarizes the allele diversity values for the repetitive regions.

The D statistics are also summarized in Tables 6 and 7. No Tajima's D statistics were significant, suggesting that the *bnb* locus is not under strong selection and is therefore an appropriate locus to use for comparison to the X chromosome. The D* statistics involving the Gombak combined X chromosome sequence were significant, as was the separate analysis for the *crc* locus. However, because there was zero sequence variability on the X^{D} chromosomes it was not possible to calculate D statistics for those sequences.

The HKA test between the Gombak X^{D} and X^{ST} samples was significant ($\chi^2 = 5.88$, $P = 0.015$), showing evidence for selection on the X^{D} chromosome. Table 8 shows the observed and expected values from the test, which indicate a lack of polymorphism within the X-linked sequence of driving males relative to their autosomal sequence, and excess divergence between X^{ST} and X^{D} linked sequence relative to the autosomal sequence. The HKA test between the Gombak and Soraya samples was not significant ($\chi^2 = 0.044$, $P = 0.833$). This means the test failed to detect either a disparity in polymorphism between the X-linked and autosomal sequence or a disparity in divergence between Gombak and Soraya at the X-linked and autosomal loci. The results of the K^*_{ST} tests (Table 9) show significant divergence, which persists after Bonferroni correction, between the groups of flies for three of the four loci and both sets of pooled sequences.

Phylogenetic analysis of the X-linked sequence (Figure 8a) reveals that all Gombak X^D sequences form a single cluster, as expected from the lack of variation among those males. While one Gombak X^{ST} sequence was similar to the X^D sequences, no males producing an unbiased sex ratio had X chromosome sequence identical to the group of X^D sequences. Thus, there were apparently no males in this sample carrying suppressed drive. Before the outgroup method was used to root the tree, it was evident that Soraya and Gombak formed monophyletic groups (unrooted tree not shown). The three Soraya sequences which are similar to one another but different from the remainder of the group can be easily identified in Figure 6 (labeled Sor6, Sor7, and Sor12). In contrast to the X chromosome sequence, phylogenetic analysis of the autosomal sequence (Figure 8b) fails to recover distinct groups. Autosomal sequences from driving and non-driving males are intermingled on the branches of the tree, and even flies from the two populations did not fully resolve into separate clades.

Discussion

In this study I identify a striking pattern of sequence polymorphism and divergence associated with X chromosome drive in *Cyrtodiopsis* stalk-eyed flies. The sampled X^D chromosomes have zero sequence polymorphism, while Gombak X^{ST} chromosomes, Soraya X chromosomes, and autosomal regions from all three groups contain abundant polymorphism. Remarkably, even the repetitive regions of the X^D chromosome exhibit zero polymorphism. The possible explanations for this dramatic result fall into two categories: demography or selection. One demographic explanation, a

population bottleneck, could cause low variability, because there can only be as many alleles at a locus as there are chromosomes. However, a bottleneck should affect the entire genome, and certainly more than just one class of X chromosome. Thus, a historical bottleneck in the wild is an unlikely explanation. Because the Soraya flies were wild-caught but the Gombak flies had spent approximately 20 generations in the lab, less sequence polymorphism in Gombak might be expected as a consequence of recent reduced population size. The evidence regarding this prediction is somewhat mixed. On the one hand, the Soraya flies appear to harbor more autosomal polymorphism than the Gombak flies, but on the other hand X-linked polymorphism is similar between the Soraya and Gombak X^{ST} samples. Repeat number variability is also similar in the Soraya and Gombak X^{ST} samples. Therefore, while it is possible that maintaining the Gombak population in the lab has reduced genetic variability, that effect has been minor. Like a historical bottleneck, establishing a laboratory population seems unlikely to account for the complete loss of variability in only one class of X chromosome.

Another demographic factor to consider is the effective population size of the X chromosome relative to the autosomes. In a population with an unbiased sex ratio there will be four autosomes in each pair for every three X chromosomes and the autosomes should, therefore, support more genetic diversity. Some of the polymorphism data in this study are consistent with this prediction, as Soraya autosomal sequence is more variable than Soraya X chromosome sequence. However, the data from the Gombak X^{ST} flies are not consistent with the prediction, as there are similar levels of polymorphism in the autosomal and X-linked sequences. An interesting point to consider is that when X chromosome meiotic drive invades a population the relative proportion of X

chromosomes actually increases; for example, in a population that is 60% female, X chromosomes are four fifths as frequent as autosomes, not three quarters. This change in frequency brings the X chromosome closer to autosomes in the ability to support genetic variation. Because wild populations of *C. dalmanni* are female-biased (Wilkinson and Reillo 1994), reduced effective population size of the X chromosome should be less of a factor in this species than in species with unbiased population sex ratios (cf. Wright et al. 2004).

Instead of demographic processes, selection provides a better explanation for the dramatic lack of polymorphism on the Gombak X^D chromosome. The HKA test revealed evidence for selection on the Gombak X^D when compared to the Gombak X^{ST} chromosome. When contrasted with the result of the HKA test performed on the Gombak X and Soraya X sequence, it is apparent that selection has been concentrated on the chromosome that carries meiotic drive. By itself, however, this result does not rule out or confirm any one type of selection. Further analysis must be performed to distinguish between the different forms of selection, but ironically, having zero polymorphism instead of merely very low polymorphism makes statistical analysis to differentiate between different types of selection more difficult or even impossible.

When there is zero polymorphism, it is not possible to calculate Tajima's or Fu and Li's D* statistics. Thus, I could only calculate those statistics for the Gombak X^{ST} and Soraya samples. Fu and Li's D* statistic is designed to detect and differentiate between background and balancing selection (Fu and Li 1993), with a positive value indicating balancing selection and a negative value indicating background selection. The significant D* statistics (Tables 6 and 7) all involved the Gombak X^{ST} sequence. The

significant statistics were positive, which suggests evidence for balancing selection on the Gombak X chromosome and no evidence of background selection. The presence of balancing selection supports the prediction made by a female-biased population sex ratio (Wilkinson and Reillo 1994) that a force operates counter to the natural tendency of an X^D chromosome to sweep to fixation (Wilkinson et al. 2006).

A second way to differentiate between forms of selection is to examine the allele frequency distribution. The H test (Fay and Wu 2000), which is designed to identify hitchhiking events, tests for the characteristic deficiency of intermediate-frequency alleles that is created by positive selection. Like Fu and Li's D*, the H test is not possible to conduct when there is zero polymorphism. Therefore, while it is intuitively clear that because there is only one X^D haplotype there is a deficit of intermediate-frequency alleles in the X chromosome sequences from drive-carrying males, I cannot support that assertion with a *P* value. Such a severe lack of variability is unlikely to be due to background selection, which is predicted to leave behind an allele frequency distribution that is difficult to distinguish from the neutral expectation (Charlesworth et al. 1995). In addition, the presence of only one allele is unlikely to be due to balancing selection, which selects for diversity rather than a single allele. Therefore, the evidence suggests that positive selection has played the biggest role in shaping patterns of polymorphism and divergence on the X^D chromosome, with a possible contribution from balancing selection but little evidence to suggest a role of background selection. Unlike what was found by Dyer et al. (2007), the presence of only a single drive haplotype in this study suggests the most recent selective sweep on the *C. dalmanni* X^D chromosome must have been recent enough that no variation has since accumulated.

A major obstacle to the inference of positive selection on the *C. dalmanni* Gombak X^D chromosome is that while a selective sweep eliminates linked neutral variation, it is not predicted to have an effect on genetic divergence (Kaplan et al. 1989; Maynard Smith and Haigh 1974). However, the results of the K^*_{ST} tests and observed and expected values of the HKA test show that there is significant divergence between the X^D and X^{ST} arrangements in the Gombak population, and the neighbor-joining trees in Figure 8 show that the X chromosome sequences have assorted into monophyletic groups while the autosomal sequences are still intermingled. The usual assumption underlying theoretical work on genetic hitchhiking is that a new, favorable allele arises at single locus and increases the fitness of the organism in which it resides. But meiotic drive systems are complex, involving drive, target, modifier and suppressor loci, and evolution at one locus in effect changes the environment of the other loci. This is the essence of intragenomic conflict, which can create antagonistic coevolution similar to what occurs between predators and prey, parasites and hosts (reviewed in Kniskern and Rausher 2001), or the two sexes within a species (Parker 1979; Parker 2006). Unlike simple positive selection, antagonistic coevolution between loci is expected to promote rapid divergence between populations and possibly drive speciation (Rice et al. 2005).

Meiotic drive neatly fits the description of a system that could lead to divergence and reproductive isolation between species. Because meiotic drive is associated with sperm production, it is an attractive target for investigations into hybrid dysfunction and male hybrid sterility in particular (Frank 1991; Hurst and Pomiankowski 1991). Drive-suppressor systems have been shown to evolve rapidly within populations (Capillon and Atlan 1999; Palopoli and Wu 1996) and diverge from the standard X chromosomes of the

same species (Babcock and Anderson 1996). Wu and Beckenbach (1983) describe a "quite unexpected" level of divergence between the *sex-ratio* drive systems of *Drosophila pseudoobscura* and *D. persimilis*, despite the fact that the two species share the same chromosomal inversion that houses the drive system. My data show a loss of polymorphism that is consistent with recent, rapid change associated with a selective sweep, and divergence between X chromosomes that are unable to recombine. While these results do not show conclusively that sex chromosome meiotic drive has caused the reproductive isolation between populations of *C. dalmanni* (Christianson et al. 2005), they are consistent with that hypothesis and suggest that more detailed genetic studies of the meiotic drive system are warranted.

Table 5: Primer sequences and product sizes

Locus	Primer sequence (5' first)	Aligned product size
125	F: TGGTGTTAATGAACGAGTGACTTC F2: GAAGACTTGCATGAATGGCA F3: TGGTGTGCGTTTGCATTTAT F4: TTCATTGCATTTGCATTCG R2: AAATGGAAAATTGTGGAAGTGG R3: GCACAAAACATGGCGAAAAT R4:TGAAGAAAAATTGTATGAAATGAAAAG R5: GCCGCAGACATGACAGTAAA	460
crc	F2: ATCAAACCTTCGTCTCAGC trrF: CCAGTTCAAATTGTAACCAACG R1: GCATAGAATTCACGTATAAGCG trrR: TCGACAATTTGCATTTCACGTGC	712
bnb	F1: GAAACACCCGTAGAAGTTGTGCCAG R2: ACACGATGCGTATGTTGTGTGGGC R3: GGGGAAAACCTTAAGCCATTA	530
bnb UTR	F1: CAGAAGACCGGCAAGTAAATG SF2: TGCAAACAATGCTCAAGGAC SF3: GGACGTTTCGAGGAAAGTGA R1: GATTTTTGCGACGGTTCAAG	391

Table 6: Summary of polymorphism, divergence and tests of neutrality, loci combined by chromosome.

	All X-linked				All autosomal			
	Gom^{D}	Gom^{ST}	All Gom	Sor	Gom^{D}	Gom^{ST}	All Gom	Sor
n	9	8	17	7	20	24	44	22
S	0	11	15	10	13	13	13	28
S_i	0	0	0	6	3	2	0	6
h	1	5	6	7	N/A	N/A	N/A	N/A
π^1	0	0.49	0.51	0.33	0.40	0.42	0.43	0.89
$\theta_W{}^1$	0	0.36	0.38	0.35	0.40	0.38	0.32	0.83
D_T	‡	1.78	1.37	-0.23	-0.21	0.37	0.72	-0.16
D^*_{FL}	‡	1.52**	1.51**	-0.40	0.06	0.67	1.07	0.17

[1]: multiplied by 10^{-2}.

†: cannot be calculated because haplotype information is not known for autosomal loci.

‡: cannot be calculated because of lack of polymorphism within this subgroup.

*: $P < 0.05$, **: $P < 0.02$.

n: number of chromosomes used in the calculations.

L: number of bases in the trimmed alignment.

S: number of nucleotide polymorphisms.

S_i: number of singleton polymorphisms.

h: number of haplotypes (data not available for autosomal sequences).

π: average number of nucleotide differences between sequences.

θ_W: Watterson's (1975) estimator of the number of segregating sites between populations, reported here per base.

D_T: Tajima's D.

D_{FL}: Fu and Li's D.

Table 7: Summary of polymorphism, divergence and tests of neutrality, by individual loci.

	Locus											
	125			crc			bnb			bnbUTR		
	Gom^{D}	Gom^{ST}	Sor	Gom^{D}	Gom^{ST}	Sor	Gom^{D}	Gom^{ST}	Sor	Gom^{D}	Gom^{ST}	Sor
n	11	14	10	9	8	9	20	24	22	22	28	26
S	0	7	5	0	7	20	11	11	14	2	2	15
S_i	0	3	4	0	0	2	1	2	4	2	0	3
h	1	4	4	1	3	5	N/A	N/A	N/A	N/A	N/A	N/A
π^{1}	0	0.48	0.25	0	0.53	1.12	0.66	0.61	0.71	0.05	0.16	1.21
$\theta_W{}^{1}$	0	0.48	0.38	0	0.38	1.03	0.59	0.56	0.73	0.14	0.13	1.00
D_T	‡	0.01	-1.39	‡	1.85	0.39	0.14	0.31	-0.30	-1.51	0.39	0.21
D^*_{FL}	‡	-0.35	-1.35	‡	1.44*	1.19	0.64	0.50	-0.24	-2.11	0.82	0.21

[1]: multiplied by 10^{-2}.

†: cannot be calculated because haplotype information is not known for autosomal loci.

‡: cannot be calculated because of lack of polymorphism within this subgroup.

*: $P < 0.05$, **: $P < 0.02$.

n: number of chromosomes used in the calculations.

L: number of bases in the trimmed alignment.

S: number of nucleotide polymorphisms.

S_i: number of singleton polymorphisms.

h: number of haplotypes (data not available for autosomal sequences).

π: average number of nucleotide differences between sequences.

θ_W: Watterson's (1975) estimator of the number of segregating sites between populations, reported here per base.

D_T: Tajima's D.

D_{FL}: Fu and Li's D.

Table 8: Result of the HKA test. The numbers before the slashes are observed values, and the numbers after the slashes are the expected values

	Autosome	X
Gombak drive males vs. Gombak non-drive males		
No. segregating sites (drive only)	13 / 9.17	0 / 3.83
No. differences (drive *vs*. non-drive)	4.13 / 7.95	9.13 / 5.3
Gombak males vs. Soraya males		
No. segregating sites (Gombak only)	13/13.54	15/14.46
No. differences (Gom. vs. Sor.)	7.64/7.10	11.03/11.58

Table 9: Results of the K^*_{ST} tests. Numbers in each cell are the K^*_{ST} statistics. **: $P < 0.001$, ***: $P < 0.0001$

	ms125	*crc*	X combined	*bnb*	*bnb* UTR	Autosomal combined
Gom^{ST} males vs. Gom^{D} males	0.550***	0.383***	0.505***	0.009**	0.029	0.034**
Soraya males vs. all Gombak males	0.226***	0.254***	0.209***	0.086***	0.188***	0.078***

Figure 6. The genotype of each fly at each polymorphic site in the four DNA regions. Fly names beginning with "Sor" are from the Soraya population, those beginning with "Gom" are from the Gombak population and the laboratory screen for meiotic drive. Gombak flies with an asterisk at the end carry X^D, those without carry X^{ST}. All polymorphisms are in the order in which they appear in the alignments with the intervening non-variable sequence removed. The loci *ms125* and *crc* are X-linked, and *bnb* and the *bnb* UTR are autosomal. IUPAC nucleotide ambiguity codes, which are used to indicate heterozygous positions in the autosomal loci, are as follows: R = A or G, Y = C or T, M = C or A, K = T or G, W = T or A, S = C or G.

Figure 7. Allele diversity statistics calculated for the repetitive DNA regions. The first four in each are the allele diversity statistics corresponding to the four repetive regions in the *ms125* locus. The final bar in each set corresponds to the glutamine repeat region in the *crc* locus.

Figure 8. Neighbor-joining tree of X-linked sequences (Figure 8a) and neighbor-joining tree of autosomal sequences (Figure 8b). Sequence names beginning with "Sor" are from the Soraya population. Sequence names beginning with "Gom" are from the Gombak population laboratory drive screen. Gombak sequence names followed by an asterisk are known drive carriers, those without are known not to carry drive.

Fig. 6

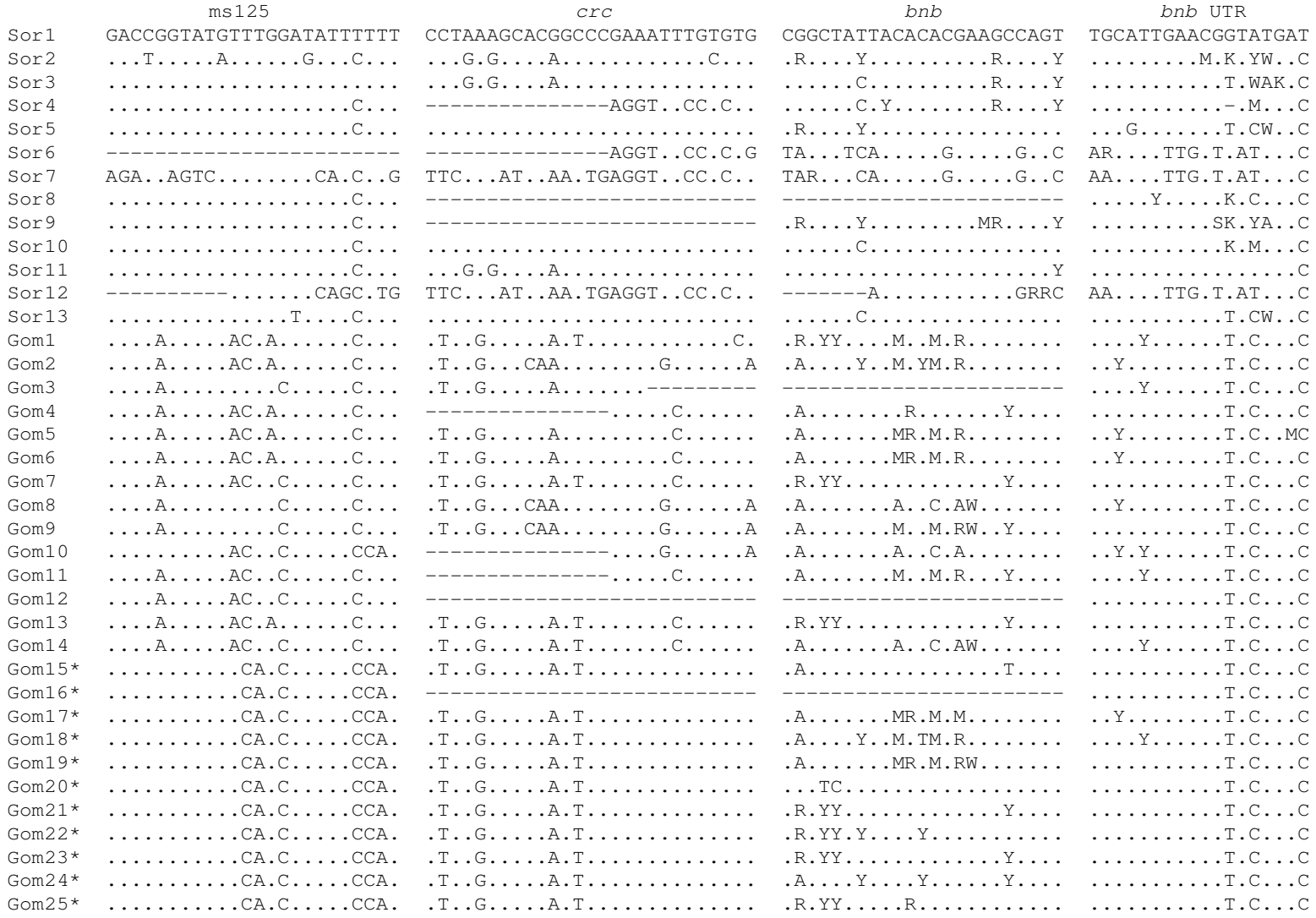

ms125
crc
bnb
bnb UTR
Sor1 GACCGGTATGTTTGGATATTTTTT CCTAAAGCACGGCCCGAAATTTGTGTG CGGCTATTACACACGAAGCCAGT TGCATTGAACGGTATGAT
Sor2 ...T.....A......G...C... ...G.G....A...........C... .R....Y.........R....Y .........M.K.YW..C
Sor3 ....G.G....A..... ......C.........R....Y ...........T.WAK.C
Sor4 .....C... ----------AGGT..CC.C.. ......C.Y........R....Y ...........-.M...C
Sor5 .....C... ..... .R....Y..... ...G.......T.CW..C
Sor6 ---------- ----------AGGT..CC.C.G TA...TCA.....G.....G..C AR....TTG.T.AT...C
Sor7 AGA..AGTC........CA.C..G TTC...AT..AA.TGAGGT..CC.C.. TAR...CA.....G.....G..C AA....TTG.T.AT...C
Sor8 .....C... ---------- ---------- .....Y.....K.C...C
Sor9 .....C... ---------- .R....Y.........MR....Y ..........SK.YA..C
Sor10 .....C... ....C..... ...........K.M...C
Sor11 .....C... ...G.G....A..... .....Y C
Sor12 ----------.......CAGC.TG TTC...AT..AA.TGAGGT..CC.C.. -------A..........GRRC AA....TTG.T.AT...C
Sor13 ..............T....C... ....C..... ...........T.CW..C
Gom1 ....A.....AC.A......C... .T..G.....A.T...........C. .R.YY....M..M.R..... ....Y......T.C...C
Gom2 ....A.....AC.A......C... .T..G...CAA........G......A .A....Y..M.YM.R..... ..Y........T.C...C
Gom3 ....A.........C.....C... .T..G.....A.......--------- ---------- ....Y......T.C...C
Gom4 ....A.....AC.A......C... ----------.....C..... .A........R.......Y.... ...........T.C...C
Gom5 ....A.....AC.A......C... .T..G.....A.........C..... .A.......MR.M.R..... ..Y........T.C..MC
Gom6 ....A.....AC.A......C... .T..G.....A.........C..... .A.......MR.M.R..... ..Y........T.C...C
Gom7 ....A.....AC..C.....C... .T..G.....A.T.......C..... .R.YY.............Y.... ...........T.C...C
Gom8 ....A.........C.....C... .T..G...CAA........G......A .A.......A..C.AW..... ..Y........T.C...C
Gom9 ....A.........C.....C... .T..G...CAA........G......A .A.......M..M.RW..Y.... ...........T.C...C
Gom10 ..........AC..C.....CCA. ----------....G......A .A.......A..C.A..... ..Y.Y......T.C...C
Gom11 ....A.....AC..C.....C... ----------.....C..... .A.......M..M.R...Y.... ....Y......T.C...C
Gom12 ....A.....AC..C.....C... ---------- ---------- ...........T.C...C
Gom13 ....A.....AC.A......C... .T..G.....A.T.......C..... .R.YY.............Y.... ...........T.C...C
Gom14 ....A.....AC..C.....C... .T..G.....A.T.......C..... .A.......A..C.AW..... ....Y......T.C...C
Gom15* ..........CA.C.....CCA. .T..G.....A.T..... .A.................T.... ...........T.C...C
Gom16* ..........CA.C.....CCA. ---------- ---------- ...........T.C...C
Gom17* ..........CA.C.....CCA. .T..G.....A.T..... .A.......MR.M.M..... ..Y........T.C...C
Gom18* ..........CA.C.....CCA. .T..G.....A.T..... .A....Y..M.TM.R..... ....Y......T.C...C
Gom19* ..........CA.C.....CCA. .T..G.....A.T..... .A.......MR.M.RW..... ...........T.C...C
Gom20* ..........CA.C.....CCA. .T..G.....A.T..... ...TC..... ...........T.C...C
Gom21* ..........CA.C.....CCA. .T..G.....A.T..... .R.YY.............Y.... ...........T.C...C
Gom22* ..........CA.C.....CCA. .T..G.....A.T..... .R.YY.Y....Y..... ...........T.C...C
Gom23* ..........CA.C.....CCA. .T..G.....A.T..... .R.YY.............Y.... ...........T.C...C
Gom24* ..........CA.C.....CCA. .T..G.....A.T..... .A....Y....Y......Y.... ...........T.C...C
Gom25* ..........CA.C.....CCA. .T..G.....A.T..... .R.YY.....R..... ...........T.C...C

Fig. 7

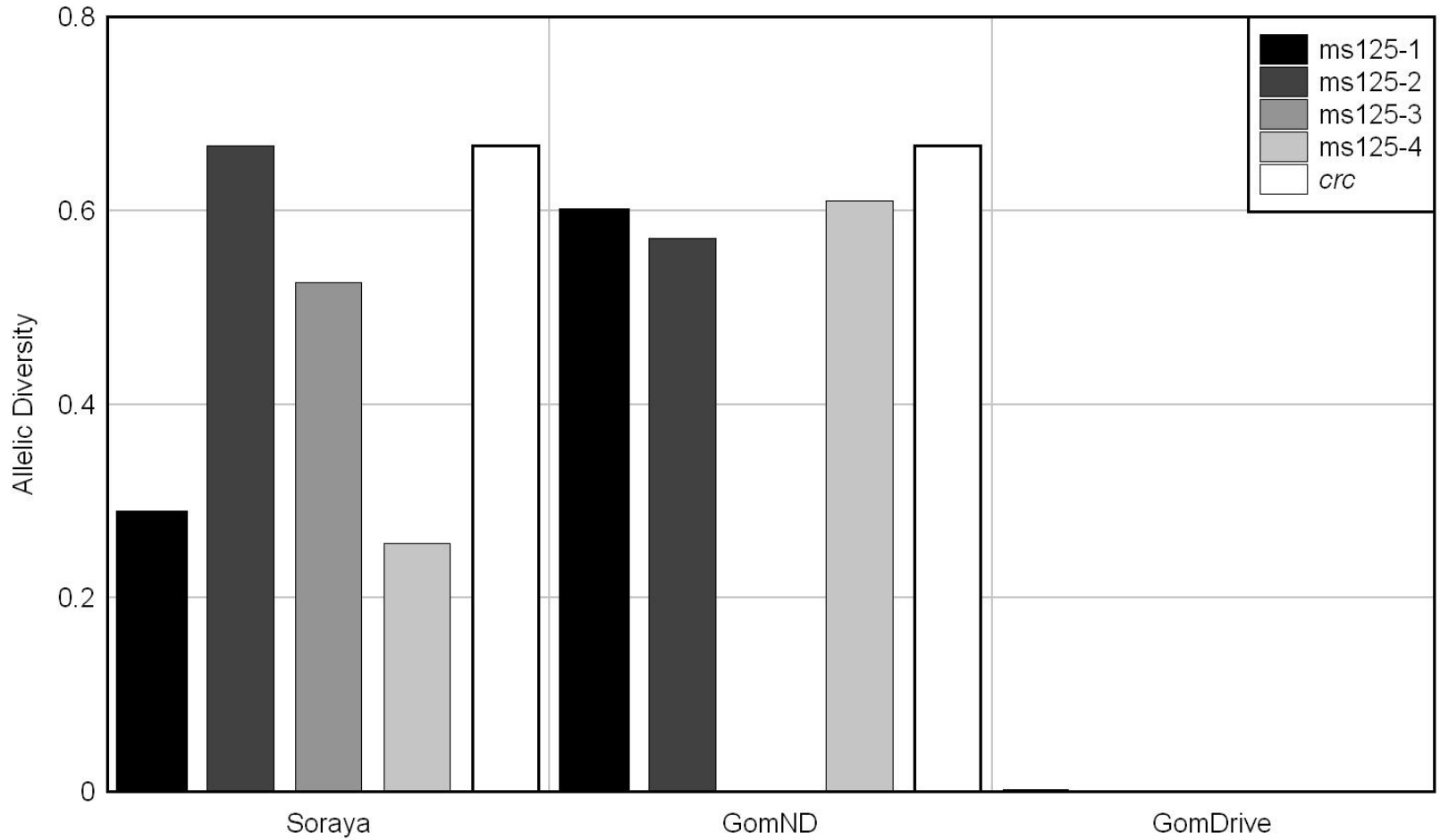

0.8
0.6
0.4
0.2
0
Allelic Diversity
ms125-1
ms125-2
ms125-3
ms125-4
crc
Soraya
GomND
GomDrive

Fig. 8a

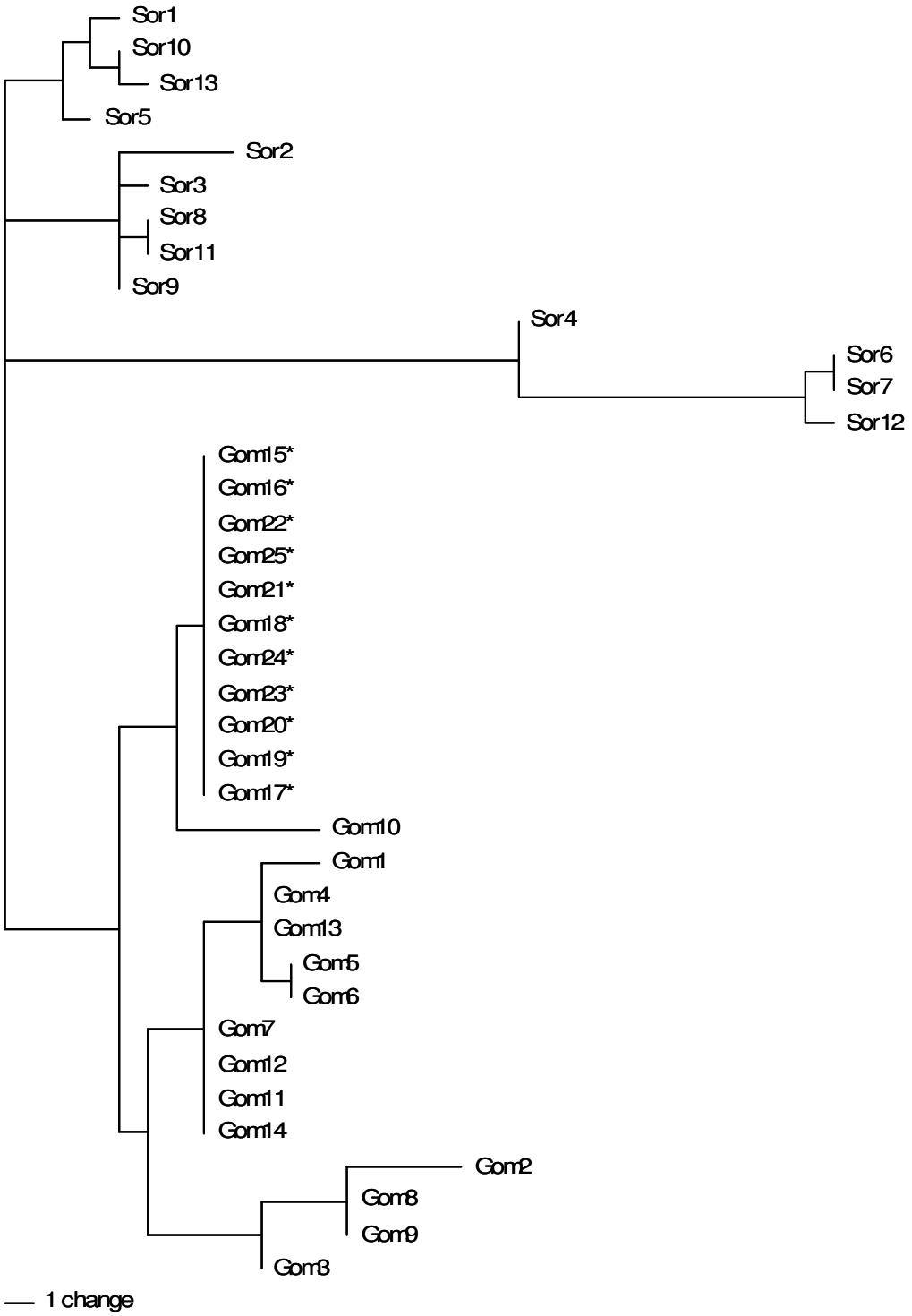
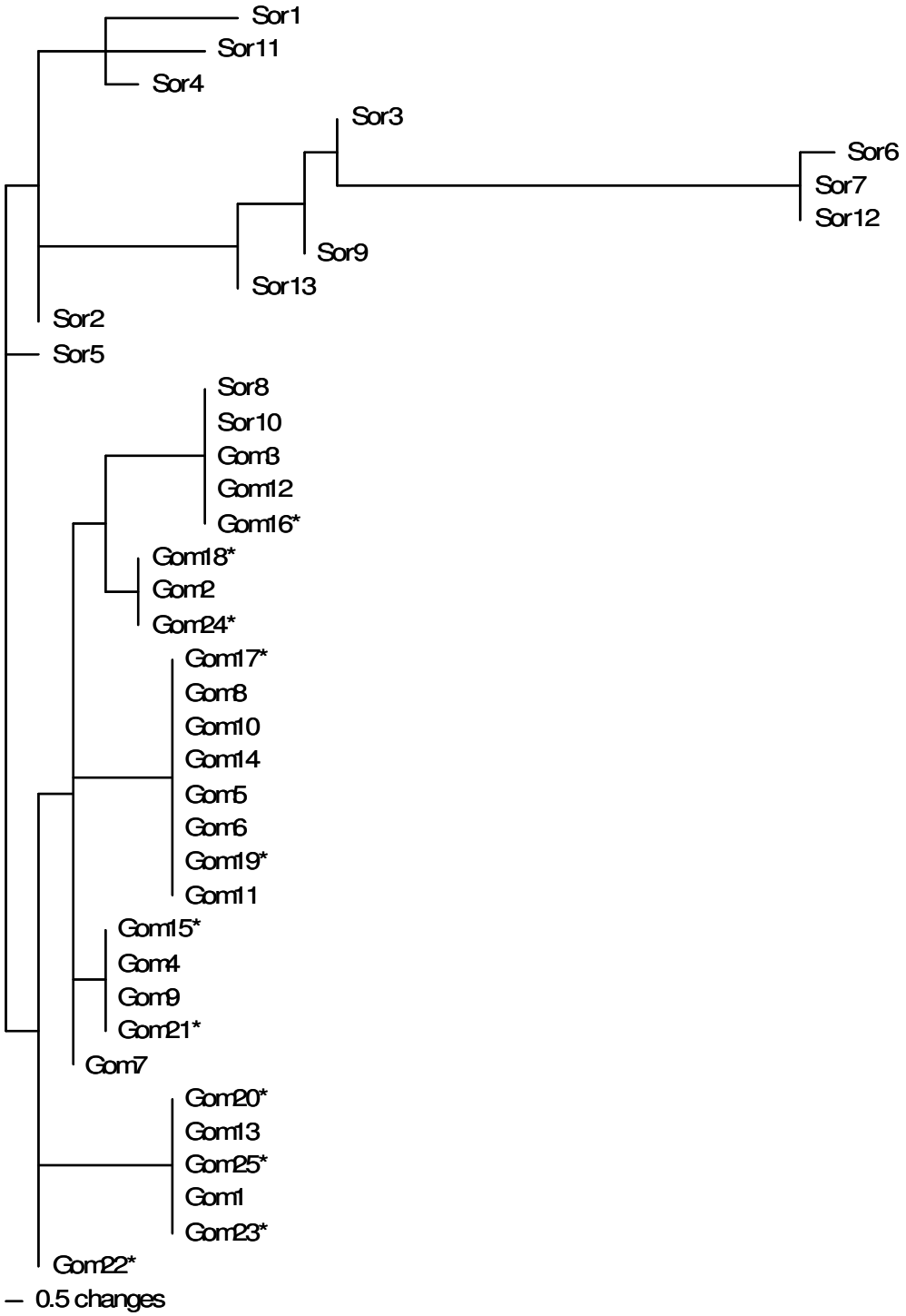

Sor1
Sor10
Sor13
Sor5
Sor2
Sor3
Sor8
Sor11
Sor9
Sor4
Sor6
Sor7
Sor12
Gom15*
Gom16*
Gom22*
Gom25*
Gom21*
Gom18*
Gom24*
Gom23*
Gom20*
Gom19*
Gom17*
Gom10
Gom1
Gom4
Gom13
Gom5
Gom6
Gom7
Gom12
Gom11
Gom14
Gom2
Gom8
Gom9
Gom3
1 change

Fig. 8b


Sor1
Sor11
Sor4
Sor3
Sor6
Sor7
Sor12
Sor9
Sor13
Sor2
Sor5
Sor8
Sor10
Gom3
Gom12
Gom16*
Gom18*
Gom2
Gom24*
Gom17*
Gom8
Gom10
Gom14
Gom5
Gom6
Gom19*
Gom11
Gom15*
Gom4
Gom9
Gom21*
Gom7
Gom20*
Gom13
Gom25*
Gom1
Gom23*
Gom22*
0.5 changes

Chapter 3: Hybrid Male Sterility and Extraordinary Progeny Sex Ratios in Stalk-eyed Flies

Abstract

I test two predictions made by the meiotic drive hypothesis of Haldane's rule. The first prediction is that loci affecting sex chromosome meiotic drive also cause male hybrid sterility. The second is that the evolution of meiotic drive has resulted in the accumulation of cryptic drive loci and suppressors of drive. I test these predictions by conducting two QTL studies on groups of hybrid males generated by crossing two genetically isolated populations of the stalk-eyed fly *C. dalmanni*. I located several QTL affecting male hybrid sterility and one affecting progeny sex ratio. I found evidence for a Y-linked modifier affecting meiotic drive, an autosomal suppressor of drive, a cryptic drive locus released from suppression in hybrids, and X-linked hybrid inviability. I discuss these results in the context of the meiotic drive hypothesis of Haldane's rule, including avenues for future research.

Introduction

A major challenge to those who study speciation is to determine the causes of early reproductive isolation between incipient species. A productive avenue for gaining

insight into this issue has been to investigate the causes of Haldane's rule (Haldane 1922). Haldane's rule states that when closely related species hybridize, the heterogametic sex of offspring are more likely to be sterile or inviable than the homogametic sex. This phenomenon is believed to be a nearly ubiquitous phase of early speciation and has been observed in all animal taxa that have been studied (Orr 1997). Haldane's rule has been revealed to have multiple causes, and it is clear that genetic factors, such as the type of sex determination system, cause different taxa to follow different paths to unisexual hybrid dysfunction (Presgraves and Orr 1998; Turelli 1998).

Two of the best-supported explanations for Haldane's rule are the dominance theory and faster-male theory. The dominance theory (Muller 1942; Orr 1993) states that if genes causing hybrid dysfunction are at least partially recessive, the heterogametic sex will be affected more than the homogametic sex. This result is expected because the degenerate (or missing, in XO taxa) sex chromosome possessed by the heterogametic sex fails to mask recessive alleles present on its homolog. The dominance theory is generally applied to cases of Haldane's rule for inviability, and applies to all taxa with a degenerate sex chromosome, whether male- or female-heterogametic. The second hypothesis, known as the faster-male theory (Wu et al. 1996; Wu and Davis 1993), applies specifically to Haldane's rule for sterility in male-heterogametic taxa, but includes taxa where the chromosome that determines maleness is not degenerate (as in *Aedes* mosquitoes, Presgraves and Orr 1998). This hypothesis states that male-specific sterility factors accumulate faster than female-specific factors, possibly because sexual selection causes more rapid change in male-specific reproductive loci. Thus Haldane's rule can result

from at least two processes: a fundamental difference between sex chromosomes and autosomes and different rates of evolution in the two sexes.

In 1991, two publications independently proposed a third hypothesis to explain Haldane's rule: the divergence of sex chromosome meiotic drive systems in isolated populations causes hybrid dysfunction (Frank 1991; Hurst and Pomiankowski 1991). Sex chromosome meiotic drive refers to non-Mendelian passage of X and Y chromosomes into gametes and causes biased progeny sex ratios. The drive hypothesis for Haldane's rule is similar to the dominance theory in that it assumes that interacting loci cause incompatibilities (Dobzhansky 1937a; Muller 1940; Muller 1942; Orr 1995), but in the drive hypothesis the loci influence meiotic drive. The system of interacting loci which causes meiotic drive has been shown to evolve rapidly within (Capillon and Atlan 1999; Palopoli and Wu 1996) and between populations (Wu and Beckenbach 1983), as also predicted by the faster-male theory. However, unlike the faster-male theory, the drive hypothesis can apply to either male- or female-heterogametic taxa (Tao and Hartl 2003) because there are two different mechanisms for producing meiotic drive: genic drive and chromosomal drive. In genic drive, loci attack their counterparts on homologous chromosomes and kill the sperm in which they are carried, which could lead to Haldane's rule in male-heterogametic species. In chromosomal drive, centromeres compete to reach the ovum during meiosis instead of being relegated to a polar body (Henikoff et al. 2001; Henikoff and Malik 2002), which in female-heterogametic species could lead to Haldane's rule. The drive hypothesis has, therefore, the potential to apply to a diversity of taxa, but few studies have yet been designed to evaluate its plausibility.

The meiotic drive hypothesis of Haldane's rule was largely dismissed at first (Coyne et al. 1991; Coyne and Orr 1993; Johnson and Wu 1992), but has since stimulated interest in the relationship between selfish genetic systems and speciation. Meiotic drive systems have been found in a wide variety of animal taxa (summarized in Hurst and Pomiankowski 1991), but may be particularly common in the Diptera (Jiggins et al. 1999). Largely due to work on several *Drosophila* species, evidence is mounting that the evolution of drive systems may be a factor in the accumulation of postzygotic isolation. Several studies have revealed meiotic drive in hybrids between *Drosophila* populations that show no segregation distortion on their own (Dermitzakis et al. 2000; Mercot et al. 1995; Tao et al. 2001; Yang et al. 2004). Other work has shown hybrid incompatibility QTLs to be associated with transmission ratio distortion in tomato (Moyle and Graham 2006). A hybrid sterility locus maps closely to the location of a meiotic drive locus in one *Drosophila* cross (Tao et al. 2001) and sterility and drive are associated with the same regions of the X chromosome in another (Orr and Irving 2005). Finally, a recent theoretical model shows the feasibility of meiotic drive leading to the evolution of hybrid sterility (Adams 2005).

The stalk-eyed fly genus *Cyrtodiopsis* presents an excellent system in which to further investigate sex chromosome meiotic drive and its effect on hybrid dysfunction. The phylogenetic relationships among multiple captive populations of the species *C. dalmanni* and *C. whitei* have been discovered (Swallow et al. 2005), and several pairs of populations have been examined for the nature and severity of reproductive isolation between them (Christianson et al. 2005). The population comparisons were chosen to represent a wide range of genetic distances between captive populations (divergence for

mitochondrial gene fragments between 0.35% and 7.83%), and revealed rapid evolution of male hybrid sterility relative to other types of pre- and postzygotic isolation. Most or all of these populations carry a sex chromosome meiotic drive complex (Wilkinson et al. 2003), which is located on the X chromosome, likely within a paracentric inversion (Johns et al. 2005; Presgraves et al. 1997). Genetic information available for use with the genus is growing rapidly, and includes more than 50 microsatellite loci spread over the X chromosome and both autosomes (Wright et al. 2004) and an EST library containing over 3500 unique genes (Baker et al, unpublished data).

In this study, I tested two predictions of the meiotic drive hypothesis. The first is that loci affecting progeny sex ratio and male hybrid sterility will map to the same chromosomal locations. The second is that the evolution of meiotic drive, as discussed in chapter 2 of this dissertation, will leave behind multiple suppressors of drive and cryptic driver loci; that is, driver loci which are completely suppressed. I chose two monophyletic populations of *C. dalmanni* that are separated by approximately 2% mitochondrial DNA sequence distance (Swallow et al. 2005) and display male-specific hybrid sterility but little prezygotic isolation (Christianson et al. 2005). I created inbred lines from these populations and crossed the lines to construct families for QTL mapping. I measured male hybrid sterility and progeny sex ratio bias in hybrids, and collected microsatellite genotype data. I then used backcross-design QTL mapping techniques to detect genomic regions affecting meiotic drive, including possible suppressor or modifier genes, and male hybrid sterility loci.

I made several specific predictions about the outcome of this experiment. First, because meiotic drive in *C. dalmanni* is already known to be located on the X

chromosome (Johns et al. 2005; Presgraves et al. 1997), I predicted that the presence or absence of drive would depend on the X chromosome and on suppressors located on the autosomes or Y chromosome. Autosomal and Y-linked suppressors have been detected or inferred in several systems (Carvalho and Klaczko 1993; Cazemajor et al. 1997; Hauschteck-Jungen 1990; Johns et al. 2005; Montchamp-Moreau et al. 2001; Presgraves et al. 1997), including *C. dalmanni*. Second, if meiotic drive and male hybrid sterility are indeed related, sterility QTL will be detected on both the autosomes and X chromosome, where meiotic drive loci are predicted to be located. Because the Y chromosome can only be mapped if it recombines with the X, and because I additionally have no Y-linked genetic markers, I cannot study loci on the Y chromosome. Third, at least some sterility QTL will localize to regions containing drive QTL. Finally, drive will be more likely to segregate in families derived from a drive-carrying male. The frequency of recombination between *C. dalmanni* drive and non-drive chromosomes (designated as X^D and X^{ST}, respectively) is very low (Johns et al. 2005), such that the X^D appears to be inherited nearly as a complete unit and there exist multi-locus microsatellite haplotypes diagnostic for the X^{ST} chromosome (Wilkinson et al. 2006). Consequently, segregation of drive among individuals carrying X^{ST} chromosomes would provide evidence for cryptic drive expressed in a hybrid background, which has been observed in studies of other hybrids (Dermitzakis et al. 2000; Fishman and Willis 2005; Mercot et al. 1995; Orr and Irving 2005; Tao et al. 2001; Yang et al. 2004).

Materials and Methods

Fly-rearing Conditions

Husbandry and egg-collection procedures are as described in Christianson et al. (2005, Chapter 1 of this dissertation). Cups containing pupae were kept for one week after progeny ceased to eclose to ensure that progeny counts were complete and sex ratio data were accurate. Progeny to be used in future crosses were separated into single-sex cages prior to the time of sexual maturity, which is approximately 22 days for females and 25 days for males (Baker et al. 2003) and kept for several days past these ages before using them in crosses. Stocks of virgin females were used within a few weeks. Males varied more widely in age when used in crosses but were frozen if not used prior to reaching 5 months of age.

Creation of Inbred Parental Lines

To facilitate linkage mapping I created inbred lines from the Soraya and Gombak populations. I began the Soraya lines by isolating three females taken from the laboratory population cage. I collected male and female progeny from these females to start the inbreeding process. I similarly established one Gombak inbred line from each of two control lines from an ongoing selection experiment (Johns et al. 2005; Wilkinson 1993; Wilkinson et al. 1998b). The control lines had not been subjected to direct selection but had experienced 50 generations of reduced population size, and therefore had reduced

heterozygosity relative to the outbred lab population cage. I used full-sib inbreeding to reduce genetic variability in the Soraya and Gombak lines for 10 and 7 generations, respectively. For the QTL crosses, I chose one line from each population (Soraya 3, or S3, and Gombak 2, or G2) that exhibited high productivity and fixed allelic differences from the other population.

Creation of Experimental Populations

The crossing scheme for this experiment is outlined in Figure 9. To generate recombinant families for QTL mapping I created two different types of hybrid F1 female flies. One type (hereafter described as the "$F1^{ST}$" type) was derived from crossing a female from the S3 line with a non-driving male from the G2 lines. The other (the "$F1^{D}$" type) was derived by crossing an S3 female to a known Gombak driver male (a male expressing an extremely female-biased progeny sex ratio characteristic of X chromosome meiotic drive, found during a screen of non-inbred Gombak population males). Every F1 female therefore had one chromosome in each pair from the Gombak population and the other from the Soraya population, but the $F1^{D}$ and $F1^{ST}$ types differed in that one carried a Gombak X^{D} chromosome and the other carried a Gombak X^{ST} chromosome.

Because F1 males derived from Gombak-Soraya crosses are sterile (Christianson et al. 2005) I used a backcross experimental design. I backcrossed both $F1^{D}$ and $F1^{ST}$ females to males from both inbred lines, creating four populations of backcross progeny ($F1^{D}$xS, $F1^{D}$xG, $F1^{ST}$xS, and $F1^{ST}$xG). I tested the fertility of a preliminary group of 60 males from these four populations. I housed each male with three virgin females,

collected four food cups over two weeks from each cage, and waited for progeny to emerge from those food cups. Only two of the 60 backcross male progeny were fertile. To obtain a more balanced ratio of sterile to fertile males for mapping, I conducted a second generation of backcrosses, using the same inbred parental line as the first generation (see Fig. 9). By this method I created progeny that had more alleles derived from a parental line and therefore were more likely to be fertile.

For this second-generation backcross design, I used multiple progeny from each generation to create multiple families of flies from which to collect genotype and phenotype data. The aim of this tactic was to generate informative data for as many loci as possible throughout the genome, with the expectation that a subset of families would provide no data at each locus. In a backcross, offspring are expected to be homozygous at 50% of loci and heterozygous at the other 50%. Choosing a single fly from that generation to perform a second-generation backcross would therefore result in 50% of loci being homozygous and uninformative for genotyping in the final progeny. Using multiple backcross progeny for the second generation of backcrosses instead results in a mixture of homozygous and heterozygous families, which occur in a 3:1 ratio when averaged over all loci.

To generate the four groups of second-generation backcross male progeny I used full sisters from each of the four backcross populations. The females were housed individually, but groups of three sisters shared a mate taken from the same inbred line as their father. This method allowed me to trace the parentage of each second-generation backcross male, generate families large enough for QTL mapping, and maximize the relatedness between the male progeny. Each male parent was rotated between the

females' cages approximately five times per week until he died, and food cups were collected twice weekly for each female until she died. I collected the male progeny from this second generation of backcrosses and scored them for several phenotypes and genotypes at 27 microsatellite loci. For a full list of second-generation backcross families and the sample sizes of each, see Supplemental Tables 1 and 2 in the Appendix.

Collection of Phenotype and Genotype Data

I measured the fertility and progeny sex ratios of 699 second-generation backcross male progeny by housing each with three virgin females and collecting food cups for two weeks. I counted all progeny emerging from the food cups and calculated the sex ratio. Males producing zero progeny were scored sterile, as were two males which produced only one offspring and whose testes contained no mature sperm bundles upon dissection and microscopic examination. I tested each fertile male for departure from a 1:1 sex ratio using one-degree of freedom chi-square tests with a continuity correction (Wilkinson et al. 2003). Under that test the minimum number of progeny required to identify a biased sex ratio is five, provided all five are of the same sex. At the end of a male's two week test period he was placed in a labeled tube in a -20°C freezer before DNA extraction.

When phenotype testing was complete I pulverized the thorax plus legs of each male in an individual 1.5 mL plastic tube and extracted DNA using Qiagen DNEasy kits (Valencia, CA). Microsatellite genotype data were collected using primers designed for *C. dalmanni* (Wright et al. 2004). I amplified the microsatellite loci in 10 μL PCR

reactions on a thermal cycler (MJ Research). Each 10 μL reaction contained 1 μL of 10X PCR buffer (200 mM Tris-HCl, 500 mM KCl), 0.5 μL 50 mM $MgCl_2$, 0.25 μL 8 mM mixed dNTPs, 0.05 μL 5 units/μL recombinant Taq DNA polymerase (all of the above Invitrogen, Carlsbad, CA), 0.5 μL each of 10 μM forward (a mixture of unlabelled and 6-FAM, NED, or HEX-labelled) and reverse primers, and the remainder sterile, deionized water. PCR was started at 94°C for 2 min. followed by 32 cycles of 94°C for 30 sec., 52°C 30 sec., 72°C for 30 sec., and terminated with 72°C for 7 min. When possible, I amplified three primers in a single multiplex reaction. Reagent concentrations in multiplex reactions were identical to single-primer reactions with 0.5 μL of each forward and reverse primer, and the quantity of water was adjusted accordingly to maintain a reaction volume of 10 μL. I determined the size of amplified products using an ABI 3100 automatic genetic analyzer and the Genotyper v2.5 software package (both Applied Biosystems, Foster City, CA).

Linkage Mapping and QTL Analysis

Before attempting to locate QTL for the phenotypes of interest, I constructed linkage maps of the two autosomes and the X chromosome (Wolfenbarger and Wilkinson 2001). I used the genotypes of one or more generations of parents to unambiguously determine the population origin of each microsatellite allele and, therefore, code the data for linkage analysis. In some cases where there were no informative data from parents I was able to determine the origin of the alleles based on the pattern of segregation. Inbreeding was sufficient to ensure diagnostic microsatellite alleles at many loci;

however, it did not eliminate all heterozygosity within populations or sharing of alleles between the two populations. Flies with an allele of ambiguous origin were coded as missing data at that locus. In some families at some loci genotypes could not be unambiguously coded with regard to parental origins of alleles, but still provided useful information on the frequency of recombination between linked loci. This usually occurred with X-linked loci, which I could identify based on segregation patterns across generations, but occasionally occurred with autosomal loci whose positions were known from a previous study (Johns et al. 2005). I used these data for map construction in order to improve the precision of recombination estimates, but re-coded them as missing prior to searching for trait QTLs, as the latter process requires knowledge of the population origin of genotypes.

Linkage maps were first constructed separately for the Soraya and Gombak backcross populations using the Kosambi mapping function in Map Manager QTX v0.30 (Manly et al. 2001). After excluding family/locus combinations that were uninformative, I mapped autosomal loci using segregation expectations for a first-generation backcross. On chromosome 1 I included one locus in the Soraya families that was invariant among Gombak progeny, for respective totals of 12 and 11. On chromosome 2, the same seven loci were mapped in the Gombak and Soraya backcross families. Because the loci were in the same order in both backcrosses, I used JoinMap 3.0 (Van Ooijen and Voorrips 2001) to estimate a single set of recombination distances for only this chromosome. To map X-linked loci I used the segregation expectations for a doubled haploid population and included the same six X-linked loci in both the Soraya and Gombak backcross populations.

Prior to performing QTL analysis I tested each chromosome for an association with male hybrid sterility and biased progeny sex ratio. For each individual in the Soraya backcrosses, I surveyed each of their chromosomes for Gombak population alleles. Similarly, I counted Soraya population alleles on each chromosome of each Gombak backcross individual. I tabulated the individuals according to the presence or absence of those alleles, and presence or absence of the trait of interest (either sterility or biased progeny sex ratio). I then conducted *G* tests on each of the 2x2 contingency tables. This was a preliminary test of which chromosomes influenced each trait, and it showed whether sterile and sex-ratio biased individuals tended to carry more or fewer alleles from the backcross parent.

I used MapManager QTXb20 (freely available at www.mapmanager.org) to locate QTL affecting hybrid male sterility and sex ratio bias. In all cases, I first performed marker regression to identify markers associated with significant QTL. I then performed interval mapping on each of the three chromosomes, one at a time, separately for each backcross. When marker regression revealed a significant association on one of the three chromosomes, I selected the marker with the highest likelihood ratio statistic to set as background, and then performed composite interval mapping (CIM: Zeng 1994). Background loci were not chosen from the chromosome being mapped; therefore up to two background loci were incorporated in the generation of each map. I determined significance thresholds using permutation tests (Churchill and Doerge 1994), conducted separately for each chromosome with 10,000 permutations. I used the highest threshold ("highly significant"), corresponding to a probability of 0.001. CIM was developed for mapping continuous traits, but CIM is sufficiently robust to map binary traits accurately

with large sample sizes (Moehring et al. 2004; Moehring et al. 2006). I randomly deleted half of the flies from my sample and performed the CIM analysis on both the full and the reduced data sets and found that QTL location was not affected. This analysis indicated that my sample size was sufficient to use CIM to map the binary traits in this study (A. Moehring, pers. comm.).

Results

Linkage Maps

There were several differences between the linkage maps in the two backcrosses. Chromosome 1 was calculated to be 76.3 cM long in the Gombak backcrosses and 80.2 cM in the Soraya backcrosses; the Soraya map included one more microsatellite marker (Figure 10). Chromosome 2 was calculated to be 51.1 cM long. The X chromosome was calculated as 40.4 cM in the Soraya backcrosses but 55.3 cM in the Gombak backcrosses. Three loci on the X (ms71, *crc* and ms395) are in nearly identical locations in the two backcrosses, and the difference between the backcrosses is almost entirely due to the remaining loci. In the Gombak backcrosses, three more loci (ms106, ms167 and ms70) add 14.9 cM to the X chromosome, but in the Soraya backcrosses those same three loci segregate together 16.8 cM from ms395. Of the 438 Gombak backcross individuals with recorded genotypes at two or more of these three loci, 24 (5.5%) contained recombinant genotypes. Of the 261 Soraya backcross individuals, zero were recombinant. A *G* test on that difference is highly significant at (df = 1, $G = 21.96$, $P < 0.0001$). Two additional

microsatellite loci (ms238 and ms125) were genotyped but segregated perfectly with ms167 in both crosses.

Male Hybrid Sterility

Male hybrid fertility differed between the four backcross types. In total, 40.3% of second-generation backcross males were fertile. The number of males tested in each backcross type varied between 96 and 220, and fertility varied between 24.1% and 51.4% (Table 10). A chi-squared test failed to detect an association between backcross population and fertility (df = 1, $X^2 = 3.48$, $P = 0.06$). However, fertility was significantly associated with the drive status of the original male parent (df = 1, $X^2 = 22.09$, $P < 0.0001$). Surprisingly, the $F1^{D}$xS and $F1^{D}$xG backcrosses produced more fertile progeny than the $F1^{ST}$xS and $F1^{ST}$xG backcrosses.

Fertility was affected by both autosomes and the X chromosome. *G* tests showed a highly significant association between the presence of non-backcross parent alleles and male hybrid sterility on each chromosome in each backcross (Table 11). Statistical significance was unaffected by application of the sequential Bonferroni procedure for correction of *P* values under multiple tests (Rice 1989). In all cases, fertility was more likely when all alleles came from the backcross parent's population. In the Gombak backcrosses, at least one QTL affecting fertility was detected on chromosome 1 and on the X chromosome (Figure 11a). On chromosome 1, at the maximum likelihood ratio statistic (LRS) value, the QTL explained 14% of the variance of male hybrid fertility. At the corresponding LRS maximum on the X chromosome, that figure was 12%. In the

Soraya backcrosses, at least one sterility QTL was detected on each of chromosomes 1 and 2 (Figure 11b). On chromosome 1, the QTL explained 13% of the variance of hybrid fertility at the LRS maximum. On chromosome 2, that figure was 21%. These measures are conservative estimates of the percent variance explained by the QTL because there may be more than one QTL in the area where the LRS exceeds the "highly significant" threshold.

Progeny Sex Ratio

Progeny sex ratio varied between the four backcrosses in unexpected ways. The frequency of biased progeny sex ratios among fertile males varied between 26% and 47% in the four backcrosses, for an overall frequency of 40.4% (Table 10). In the Soraya backcross families these were nearly always male-biased broods, and in the Gombak families they were nearly always female-biased (Figure 12). Chi-squared contingency table analysis shows no significant relationship between backcross family (Soraya or Gombak) and the frequency of biased brood sex ratios (df = 1, $X^2 = 2.86$, $P = 0.09$). However, the frequency of biased brood sex ratio did vary with the drive status of the original male parent (df = 1, $X^2 = 5.46$, $P = 0.02$): the frequency of biased sex ratios produced by second-generation backcross males was higher in the $F1^D$xG and $F1^D$xS backcrosses.

Only chromosome 1 in the Gombak backcrosses had a detectable effect on progeny sex ratio. *G* tests detected an association of non-backcross population microsatellite allele ancestry with sex ratio bias on chromosome 1 in the Gombak

backcrosses, but no other cases (Table 12). These results were unaffected by application of the sequential Bonferroni procedure (Rice 1989). The presence of Soraya population alleles in this case was associated with a greater frequency of biased progeny sex ratio. QTL mapping of progeny sex ratio detected one significant QTL on chromosome 1 in the Gombak backcrosses (Figure 12). At the LRS maximum, the QTL explained 23% of the variance in progeny sex ratio. Because only fertile progeny could be used to map this trait, the sample size was reduced in comparison with the analysis of male hybrid sterility. Few fertile recombinant individuals were available in the Soraya backcross families, and consequently interval mapping failed on chromosome 2 and the X chromosome, and no QTL were detected on chromosome 1.

Selective Loss of Genotypes

Examination of marker allele frequencies indicated that not all genotypes were equally represented in the second-generation backcross progeny. First, in the Soraya backcrosses, the Gombak X^D chromosome was completely lost. While 21 males Soraya progeny carried at least one X-linked, Gombak-derived microsatellite allele (Table 11), all 21 were from the $F1^{ST}xS$ backcross. Second, while 229 Soraya backcross progeny carried intact Soraya X chromosomes (Table 11), none carried an intact Gombak X chromosome, either X^D or X^{ST}. This difference cannot be analyzed using a test of association because zero males carried intact Gombak chromosomes. Similarly, in the Gombak backcrosses, 279 males carried intact Gombak X chromosomes (Table 11), but zero fertile and only 14 sterile males carried intact Soraya X chromosomes. This

difference is highly significant according to a G test of association (df = 1, G = 22.74, P < 0.0001).

Discussion

At the start of this study, I made several predictions about the outcome of the results. Some of these predictions were supported, and some were not. In general, though, the results I obtained suggest that X chromosome drive is intimately involved in the explanation for Haldane's rule in *C. dalmanni*. First, I uncovered evidence for inviability of hybrids that carried a Gombak X^D chromosome. Consequently, I was unable to map loci on the drive chromosome. Second, males in all four backcrosses produced broods with biased sex ratios, despite the loss of the original X^D chromosome, due to the unmasking of cryptic drive. Third, two of four backcrosses produced extremely male-biased, rather than typical female-biased, broods due to a probable Y-linked modifier. Fourth, I detected QTL indicating the presence of autosomal suppressors of meiotic drive. Finally, while there was evidence for both autosomal and X-linked effects on male hybrid sterility and biased progeny sex ratio, these two traits did not map to the same chromosomal intervals. Below, I discuss the implications of these results for understanding the role of meiotic drive in stalk-eyed fly speciation.

Linkage Maps

Previous studies have provided evidence for a large X chromosome inversion or inversion complex that is associated with sex chromosome meiotic drive in *C. dalmanni* (Johns et al. 2005, Chapter 3 of this dissertation). The unexpected difference in this study between the X chromosome linkage maps in the Soraya and Gombak backcrosses indicates the presence of a second inversion. There is a significant difference between the frequency of recombinant individuals in the Soraya and Gombak backcrosses at ms70, ms106 and ms167, and as a result the Soraya X appears to be truncated with the three markers mapping to a single location. This result is consistent with an inversion polymorphism between the Soraya and Gombak populations rather than a simple artifact of disparate sample sizes. The inversion breakpoint is most likely located between ms395 and the apparent location of ms70, ms106, and ms167 on the Soraya X chromosome. Because inversion polymorphisms often suppress recombination (Sturtevant 1917; Sturtevant and Beadle 1936), and because QTL mapping requires recombination, this hypothesized inversion polymorphism constrains X chromosome mapping in this study. However, no QTL from either male hybrid sterility or sex ratio bias localized to the end of the X chromosome containing the putative inversion, so this constraint is unlikely to have limited my ability to map these traits.

Male Hybrid Sterility

QTL mapping reveals that male hybrid sterility is strongly influenced by both autosomal and X–linked factors, as predicted. Maps of the Soraya and Gombak backcrosses (Figures 11a and 11b) identified highly significant QTL on chromosome 1, although the broad peaks make it difficult to determine whether the QTL are in the same location in the two backcross groups. A QTL was also detected on the X chromosome in the Gombak backcrosses, but not in the Soraya backcrosses. However, because the presence of even a single Gombak allele on the X chromosome led to sterility in the Soraya backcross males (Table 11), it is apparent that the X chromosome has a very large effect on sterility in both backcrosses. The unexpected observation that $F1^{D}xS$ and $F1^{D}xG$ families had a higher incidence of fertility than $F1^{ST}xS$ and $F1^{ST}xG$ could be explained by inbreeding depression given that the original drive parent was an outbred, rather than inbred, male.

Extraordinary Sex Ratios

The most astonishing result of this study was the appearance of sex-ratio-biased broods in all four backcross families, counter to expectation. Because the original parent carried an X^{D} chromosome, I expected to see female-biased broods produced by the $F1^{D}xS$ and $F1^{D}xG$ backcrosses. While that prediction was borne out in the $F1^{D}xS$ backcross, the $F1^{D}xG$ progeny produced male-biased, not female-biased, broods. Similarly, $F1^{ST}xS$ progeny produced female-biased broods, and $F1^{ST}xG$ progeny

produced male-biased broods, even though I had not expected any progeny sex ratio bias from these males. Because the original X^D chromosome was lost, all X-linked alleles carried by fertile males were derived from the S3 inbred line. This observation means that the female-biased broods produced by $F1^D$xS males did not result from the original X^D chromosome. The S3 inbred line was not known to produce biased sex ratios; thus, no $F1^{ST}$xS or $F1^D$xS males carried a known X^D chromosome. Therefore, the most likely explanation for the female-biased broods in the Soraya backcrosses is that an X chromosome carries cryptic drive which is normally suppressed but is released from suppression in hybrids. A similar phenomenon was observed by Tao *et al*., (2001), who unmasked a cryptic drive after five generations of backcrossing hybrids between two *Drosophila* species. Other observations of cryptic meiotic drive have been made in *Drosophila* (Dermitzakis et al. 2000; Mercot et al. 1995; Orr and Irving 2005; Yang et al. 2004) and *Mimulus* yellow monkeyflowers (Fishman and Willis 2005), but never before in *Cyrtodiopsis*. Cryptic meiotic drive is predicted to exist in systems where drive repeatedly evolves and is in turn repeatedly suppressed.

The appearance of strongly male-biased broods also cannot be explained by the Gombak X^D chromosome, and suggests a Y chromosome effect. Weakly male-biased broods have previously been reported in *C. dalmanni* (Presgraves et al. 1997), where the authors deduced the presence of a modifying Y chromosome (Y^m) that causes male progeny sex ratio bias when paired with an X^{ST} chromosome. Although the bias did not reach the degree found in this study, where multiple broods approached 100% male progeny, the pattern is consistent in both studies. Several lines of evidence in the present study suggest the presence of a Y-linked modifier of drive. First, because the backcross

experimental design used only female hybrids, all Y chromosomes in the second-generation backcross progeny came from inbred lines. Thus a Y chromosome effect in those males would be derived from backcross fathers, as seen here, not from males from the parental generation. Second, the data do not support an effect of an X^D chromosome, as males from both the $F1^D$xG and $F1^{ST}$xG backcrosses produced male-biased broods, even though the $F1^{ST}$xG backcross was derived from a male carrying an X^{ST} chromosome. All four backcross families began with a female from the S3 inbred line, which did not exhibit a sex ratio bias during inbreeding. Thirdly, sib-mating eliminated all but one Y chromosome per inbred line in the very first generation of inbreeding. If the sole Y chromosome present in the G2 inbred line is a Y^m chromosome, the male-biasing effect of that chromosome might be expected to occur at fairly high frequencies, as seen here.

I hypothesize that the Gombak-derived Y^m chromosome interacts with Soraya-derived alleles to produce an effect not observed in either population or either inbred line. This interaction may occur in conjunction with chromosome 1, as Gombak backcross males producing male-biased broods were more likely to carry Soraya alleles on this chromosome than those males producing 1:1 sex ratios. Thus the Y chromosome and the QTL on chromosome 1 conform to my original prediction of the presence of suppressors of drive.

Male Hybrid Inviability

The low number of intact X chromosomes in backcross progeny provides strong evidence for hybrid inviability produced by an interaction of the X chromosome with the autosomal background of another population. Because many Soraya backcross progeny inherited intact (nonrecombinant) Soraya X chromosomes, it is likely that intact Gombak chromosomes were also being transmitted at meiosis but the zygotes or embryos carrying them failed to survive. Similarly, many Gombak backcross progeny inherited intact Gombak X chromosomes and few inherited intact Soraya chromosomes, likely because the latter individuals failed to survive. All alleles from the Gombak X^D were completely lost in the $F1^D$xS backcross, which may be due to the drive-associated inversion which effectively requires that all or none of the X-linked alleles in a hybrid male come from that chromosome. This hybrid inviability is surprising because a previous study of *C. dalmanni* (Christianson et al. 2005, Chapter one of this dissertation) found little evidence for decreased progeny production of Soraya-Gombak hybrids relative to within-population values, suggesting little to no hybrid inviability. The different outcomes may be due to the autosomal genetic makeup of hybrids in the two studies. The 2005 study involved F1 hybrids, which are heterozygous at all autosomal loci, but the current study involves backcross progeny, which can be homozygous at autosomal loci. This difference means recessive X-autosome interactions may be expressed in hybrid males in the current study that were not expressed in the F1 hybrid males from the 2005 study.

In this study I uncovered hybrid inviability related to the interaction of the X chromosome with another population's autosomal background. It was originally hypothesized that sex chromosome meiotic drive could cause Haldane's rule for inviability as well as sterility (Frank 1991), although that possibility has received little attention since that time. The current study was not designed to look for hybrid inviability, and because I did not study the female backcross progeny I cannot determine whether the inviability was sex-specific, i.e. conformed to Haldane's rule. I also do not know how many hybrid inviability loci are on each chromosome. A recent detailed study of *Drosophila* found that while hybrid male sterility is disproportionately affected by the X chromosome, no such bias was detected for hybrid inviability (Masly and Presgraves 2007). The observation of a strong inviability effect of the X chromosome therefore deserves further investigation, as this is possible evidence for a little-examined aspect of the meiotic drive hypothesis of Haldane's rule.

The meiotic drive hypothesis predicts that the same loci should cause both male hybrid sterility and biased progeny sex ratios. However, to observe a one-to-one correspondence between genes affecting the two traits, it is necessary to capture incipient species when very few genetic changes have taken place to cause hybrid sterility. Once populations have already achieved postzygotic isolation, additional divergence need no longer produce perfect correspondence between loci affecting meiotic drive and hybrid sterility. Therefore, when studying populations with demonstrated postzygotic isolation, it is only predicted that some loci affecting the two traits will be the same.

While there is little evidence to support the prediction that the two traits would map to the same chromosomal regions, there is not enough evidence to effectively contradict it either. I had little statistical power to map QTL affecting progeny sex ratio in the Soraya backcrosses, which means I cannot determine if loci affecting drive and Haldane's rule are located in the same place in that population. In the Gombak backcrosses, while neither trait showed evidence of a significant QTL on the second chromosome, highly significant QTL affecting both meiotic drive and male hybrid sterility were identified on chromosome 1. This result is consistent with a previous QTL analysis (Johns et al. 2005), which concluded that a similar region of the Gombak first chromosome was the likely location of a suppressor of meiotic drive. In this study, the peaks for each QTL on chromosome 1 were broad, encompassing approximately half of the chromosome. The QTL for the two traits appeared to be centered on opposite ends of chromosome 1; therefore, the two traits are most likely not being affected by the same gene. The X chromosome presents an intriguing situation. A significant QTL affecting male hybrid sterility was detected in an area shown by other work to be part of a large inversion polymorphism containing the meiotic drive complex (Johns et al. 2005, chapter 3 of this dissertation). Because I was unable to localize X-linked loci affecting meiotic drive in the present study, I cannot conclude whether the same X chromosome interval affects both drive and sterility. However, because the two traits are bound by an inversion of the X^D relative to the X^{ST} chromosome, the QTL affecting the two traits would be nearly perfectly linked in a cross where the X^D chromosome persists.

I also tested the prediction that the evolution of drive leads to the accumulation of cryptic driver loci and suppressors of drive. As discussed in chapter 2, meiotic drive is

believed to experience repeated, intense selective sweeps as suppressors and drivers evolve in competition with one another (Derome et al. 2004; Dyer et al. 2007; Reed et al. 2005). Many of these results are consistent with this prediction, and lead to excellent opportunities to further study the link between sex ratio bias and male hybrid sterility in *Cyrtodiopsis*. First, I discovered evidence of autosomal suppressors in both the Soraya and Gombak backcrosses. Second, in the Gombak population, there is evidence of a Y-linked modifier that interacts with the Soraya genetic background to produce severe male bias in the progeny sex ratio. No Y-linked genetic markers have yet been identified in *C. dalmanni* that would enable chromosome identification, but the search for new markers is ongoing. This result provides a greater impetus to continue that search, in particular to find markers on the Y chromosome. The third, and arguably most important, result in support of this prediction was the discovery of cryptic meiotic drive. The meiotic drive hypothesis was rejected early in its history because there was no evidence of cryptic drive being unmasked in hybrids (Charlesworth et al. 1993a; Coyne and Orr 1993; Johnson and Wu 1992). The recent surge of interest in the hypothesis has been stimulated by the discovery of such cryptic drive systems in several *Drosophila* crosses (Dermitzakis et al. 2000; Mercot et al. 1995; Orr and Irving 2005; Tao et al. 2001). Cryptic drivers confirm an important assumption of the meiotic drive hypothesis, which is that drivers and suppressors undergo repeated cycles of evolution. With a large number of *C. dalmanni* ESTs under development, more detailed mapping and study of the active and cryptic drivers and their suppressors will soon be possible.

Table 10: The four backcross types, number of males tested, and rates of sterility and biased sex ratio. In the first column, the letters in the parentheses indicate the population origins of the original two parents, with the male parent listed first. G indicates a fly from the G2 inbred line, and S indicates a fly from the S3 inbred line. $F1^{D}$ and $F1^{ST}$ represent females generated by crossing an S3 line inbred female with a drive-carrying outbred Gombak male or non-drive-carrying G2 line inbred male, respectively. Letters outside the parentheses indicate the population of the male parent used in backcrosses. Sample size refers to the number of second-generation male backcross progeny that were tested. The percent biased sex ratio is calculated out of the fertile males producing sufficient progeny for statistical analysis, not out of the total number of males tested for fertility.

Cross type	N	No. (Pct.) Sterile	No. (Pct.) Fertile	No. (Pct.) Biased
$G(G(F1^{ST}))$	220	167 (75.9%)	53 (24.1%)	20 (39%)
$G(G(F1^{D}))$	218	106 (48.6%)	112 (51.4%)	52 (47%)
$S(S(F1^{ST}))$	165	93 (56.4%)	72 (43.6%)	16 (26%)
$S(S(F1^{D}))$	96	51 (53.1%)	45 (46.9%)	19 (45%)

Table 11: The number of progeny with and without non-backcross population alleles at microsatellite markers. The *P* values show the results of *G* tests on the 2x2 contingency table corresponding to each chromosome.

Non-backcross parent alleles	Chromosome 1		Chromosome 2		X Chromosome	
	Fertile	Sterile	Fertile	Sterile	Fertile	Sterile
Gombak backcrosses						
0	83	47	135	117	159	123
≥1	82	226	30	155	6	152
G	53.22		67.27		147.02	
P	< 0.0001		< 0.0001		< 0.0001	
Soraya backcrosses						
0	64	35	79	33	121	108
≥1	53	109	38	111	0	21
G	20.64		54.02		29.60	
P	< 0.0001		< 0.0001		< 0.0001	

Table 12: The number of progeny with and without non-backcross population alleles at microsatellite markers. The *P* values show the results of *G* tests on the 2x2 contingency table corresponding to each chromosome. The *G* test could not be performed in one case because no surviving males carried Gombak alleles.

Non-backcross parent alleles	Chromosome 1		Chromosome 2		X Chromosome	
	Biased	Not Biased	Biased	Not Biased	Biased	Not Biased
Gombak backcrosses						
0	21	62	59	76	71	88
≥1	53	29	15	15	3	3
G	26.54		0.39		0.07	
P	< 0.0001		0.53		0.80	
Soraya backcrosses						
0	18	46	22	57	33	84
≥1	15	38	11	27	0	0
G	0.00		0.02		N/A	
P	0.98		0.90		N/A	

Figure 9: Cross scheme for the four backcross populations. Two crosses were begun in the first generation, using either a male known to carry an X^D chromosome or a non-driving male from an inbred line. Subsequent generations in both types of crosses involve backcrosses to one of two inbred lines. G2 and S3 refer to individuals from the Gombak and Soraya inbred lines, respectively, and GX^D refers to a known drive male from the Gombak population.

Figure 10: Linkage maps of the X chromosome and two autosomes in the Soraya and Gombak populations of *C. dalmanni*. Maps of chromosome 1 and the X chromosome were created separately for the two populations because of different numbers of informative loci on each, and the map for chromosome 2 was created using combined data from both populations.

Figure 11: QTL plots of second-generation backcross hybrid male fertility. Figure 11a shows results of the Gombak backcross families, and 11b shows the Soraya backcross families. Likelihood ratio statistic values were generated using composite interval mapping in the program MapManager QTX v.0.30. Triangles on the X axis represent the location of microsatellite marker loci, and the dashed line shows the "highly significant" threshold of QTL detection, representing a probability of 0.001 (Lander and Kruglyak 1995)

Figure 12: Histograms of brood sex ratio for each of the four backcross types. Figure 12a shows the results of the Gombak backcrosses, and 12b shows the results of the Soraya

backcrosses. Black bars indicate broods with sex ratios showing a significant departure from a 1:1 sex ratio according to chi-square analysis, white bars show those with no significant departure from 1:1.

Figure 13: QTL plots of biased progeny sex ratio among fertile Gombak backcross males. Likelihood ratio statistic values were generated using composite interval mapping in the cases of the X chromosome and chromosome 2. For chromosome 1, interval mapping was used because no significant loci were detected on other chromosomes that could be used as background for composite interval mapping. Triangles on the X axis represent the location of microsatellite marker loci, and the dashed line shows the “highly significant” threshold of QTL detection, representing a probability of 0.001 (Lander and Kruglyak 1995).

Fig. 9

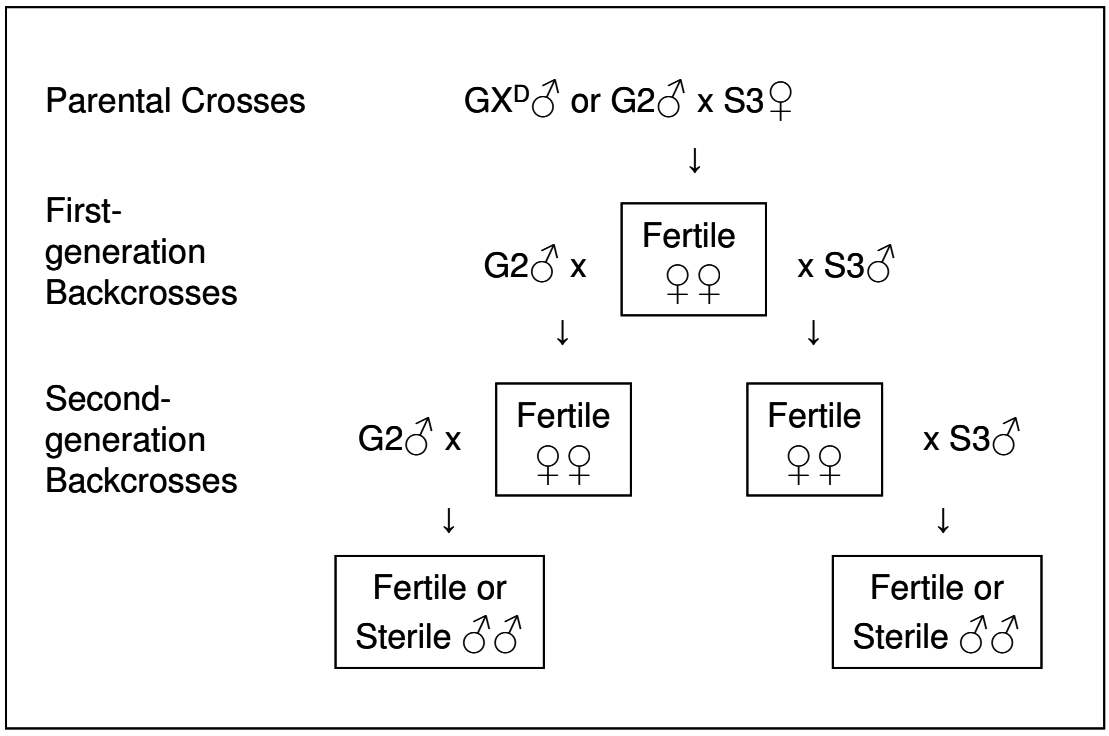
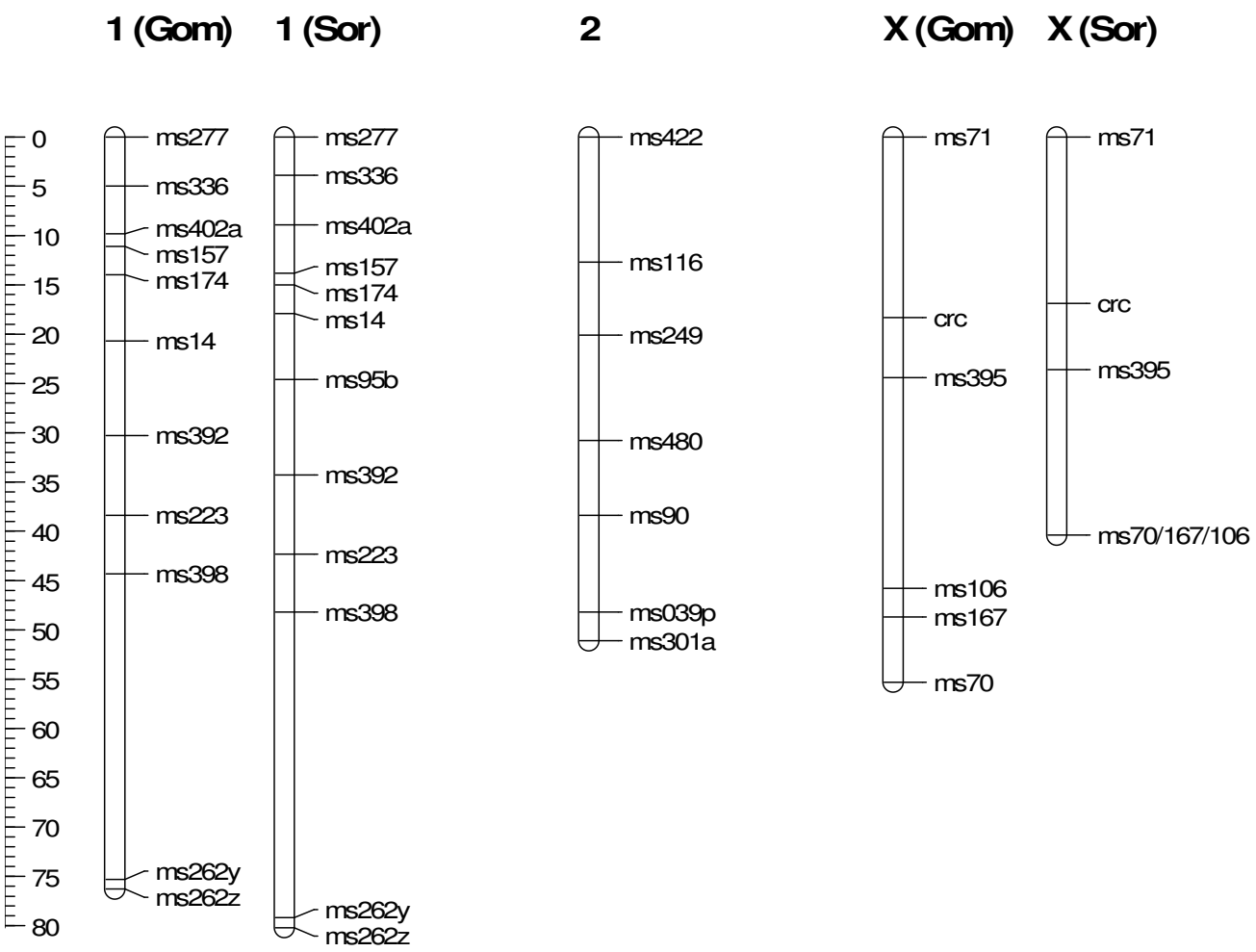

Parental Crosses
GX^{D}♂ or G2♂ x S3♀
First-generation Backcrosses
G2♂ x
Fertile ♀♀
x S3♂
Second-generation Backcrosses
G2♂ x
Fertile ♀♀
Fertile ♀♀
x S3♂
Fertile or Sterile ♂♂
Fertile or Sterile ♂♂

Fig. 10


1 (Gom)
1 (Sor)
2
X (Gom)
X (Sor)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
ms277
ms336
ms402a
ms157
ms174
ms14
ms392
ms223
ms398
ms262y
ms262z
ms277
ms336
ms402a
ms157
ms174
ms14
ms95b
ms392
ms223
ms398
ms262y
ms262z
ms422
ms116
ms249
ms480
ms90
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ms70/167/106

Fig. 11a

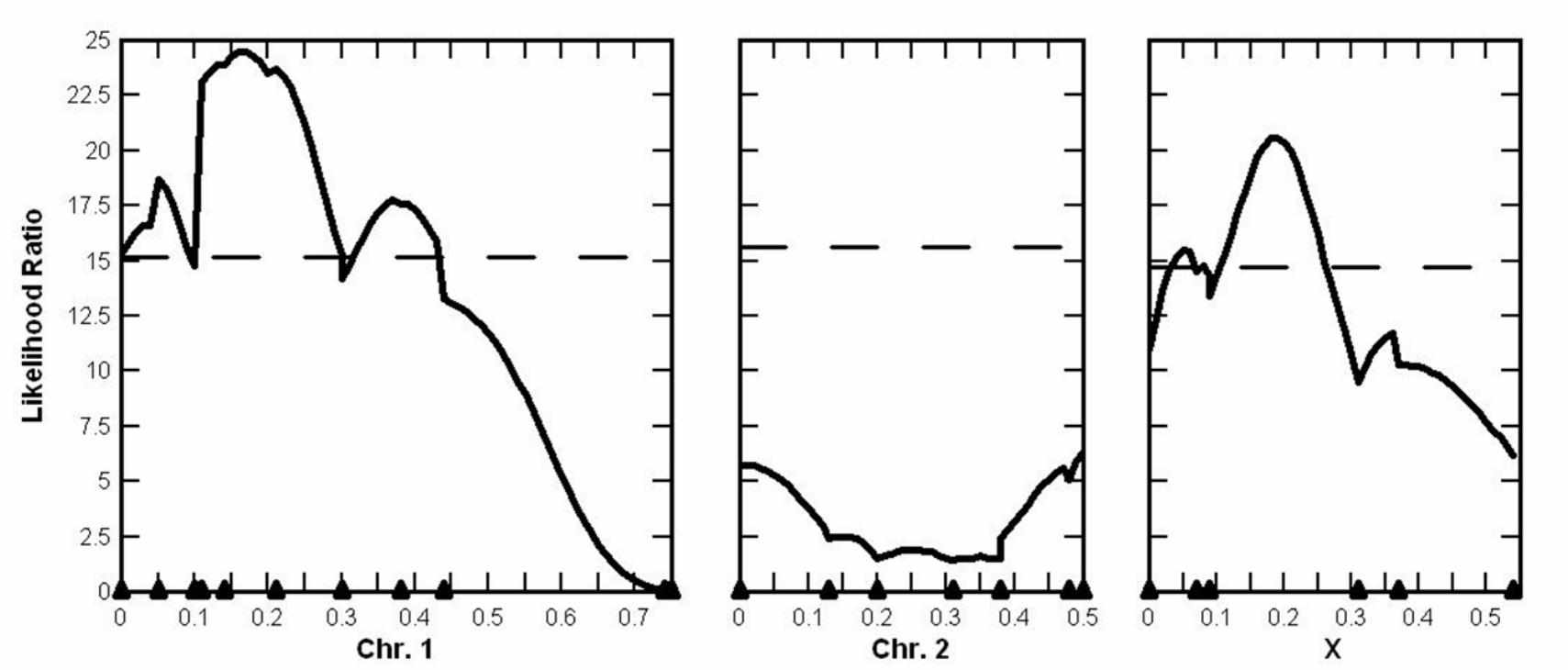

Likelihood Ratio
25
22.5
20
17.5
15
12.5
10
7.5
5
2.5
0
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7
Chr. 1
0 0.1 0.2 0.3 0.4 0.5
Chr. 2
0 0.1 0.2 0.3 0.4 0.5
X

Fig. 11b

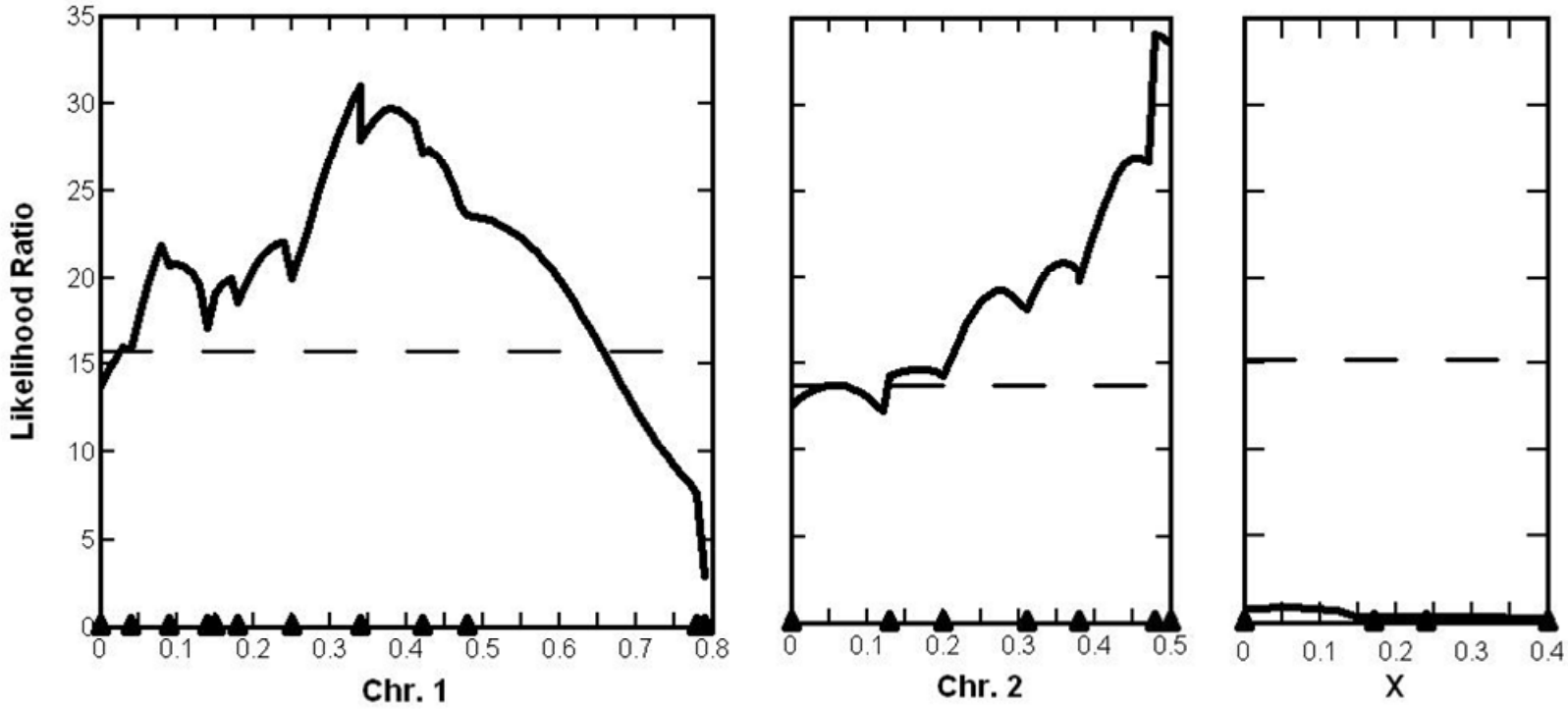

Likelihood Ratio
35
30
25
20
15
10
5
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Chr. 1
0
0.1
0.2
0.3
0.4
0.5
Chr. 2
0
0.1
0.2
0.3
0.4
X

Fig. 12a

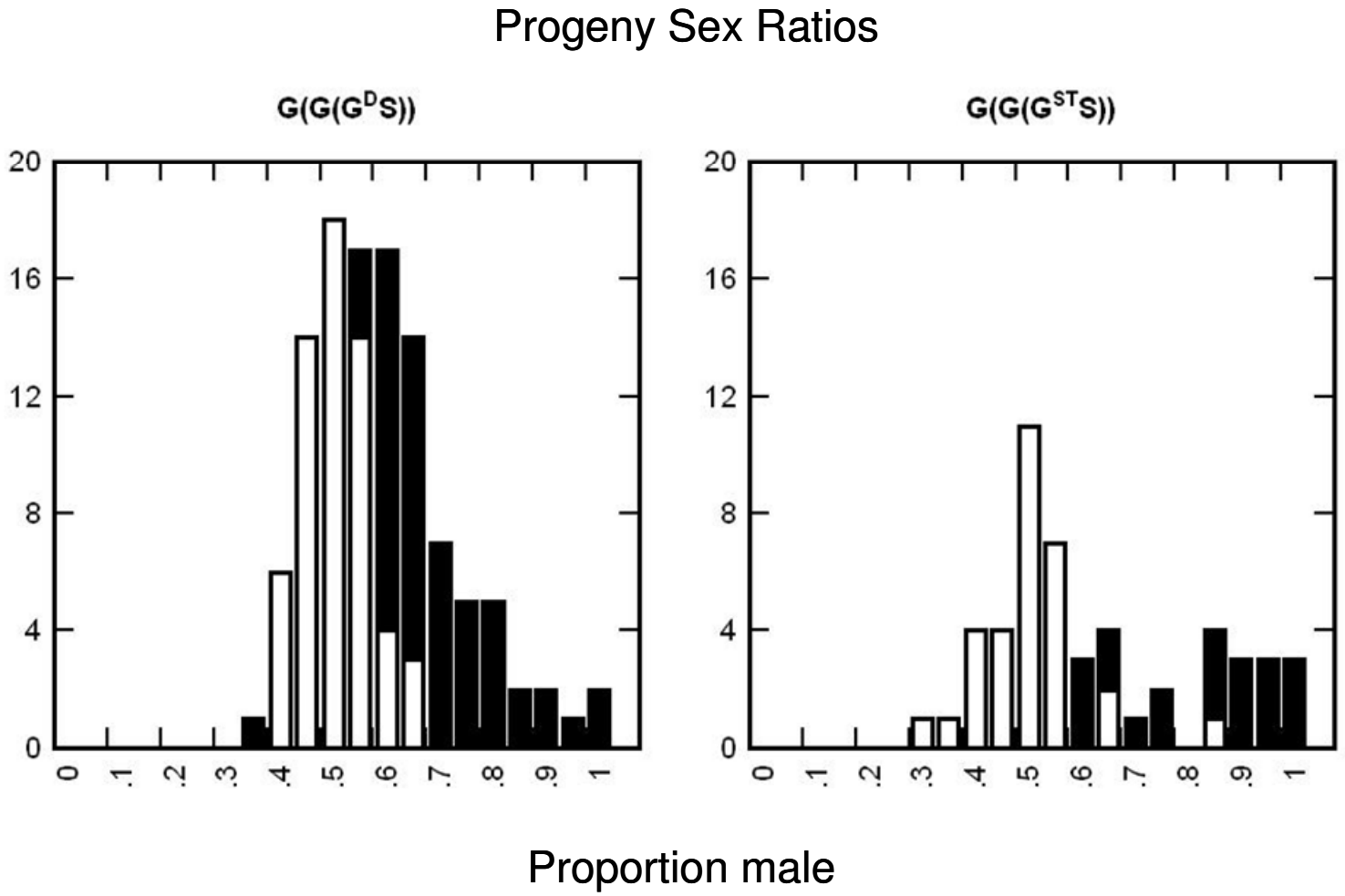

Progeny Sex Ratios
G(G(G^DS))
G(G(G^STS))
20
16
12
8
4
0
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1
Proportion male

Fig. 12b

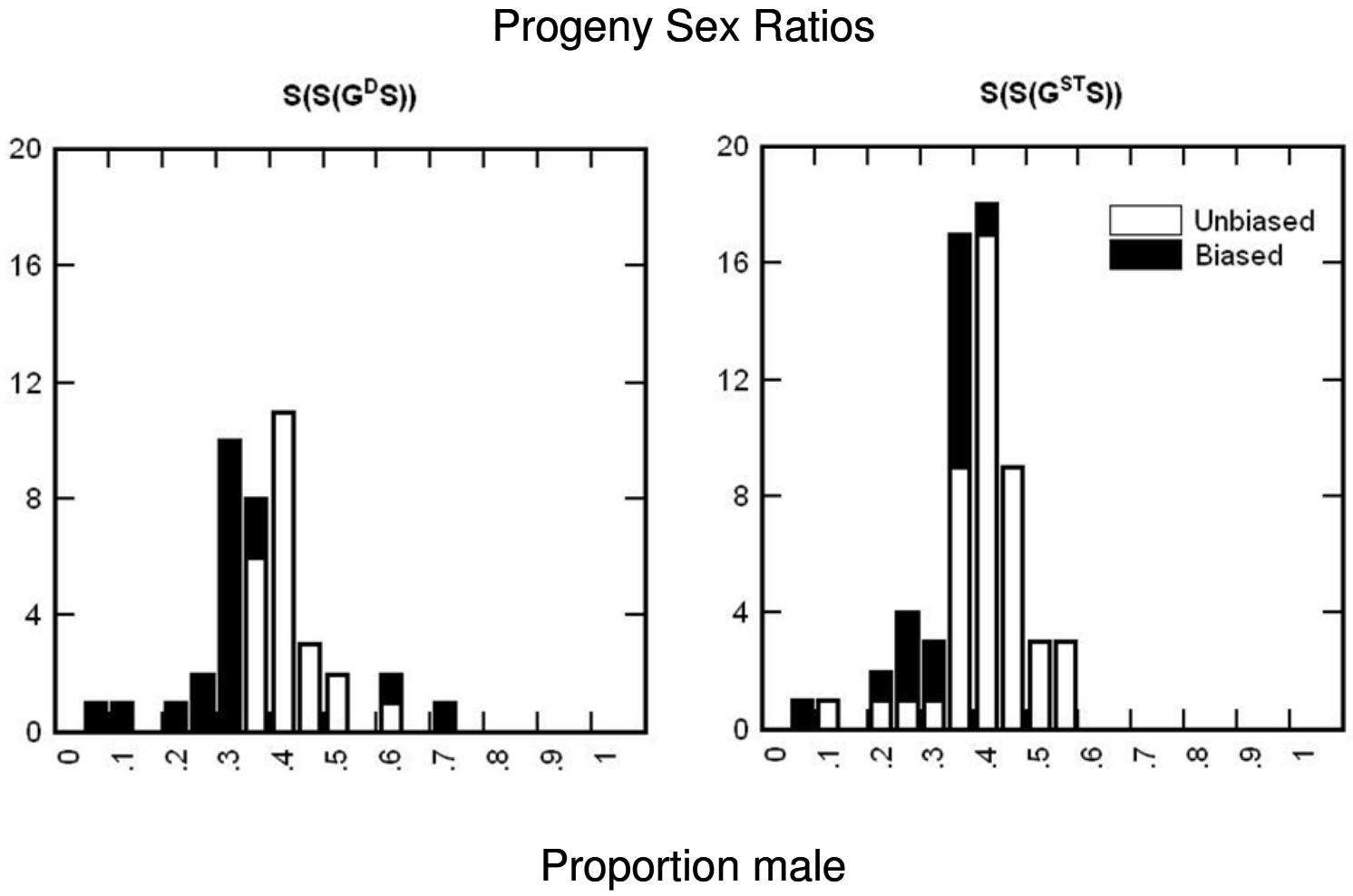
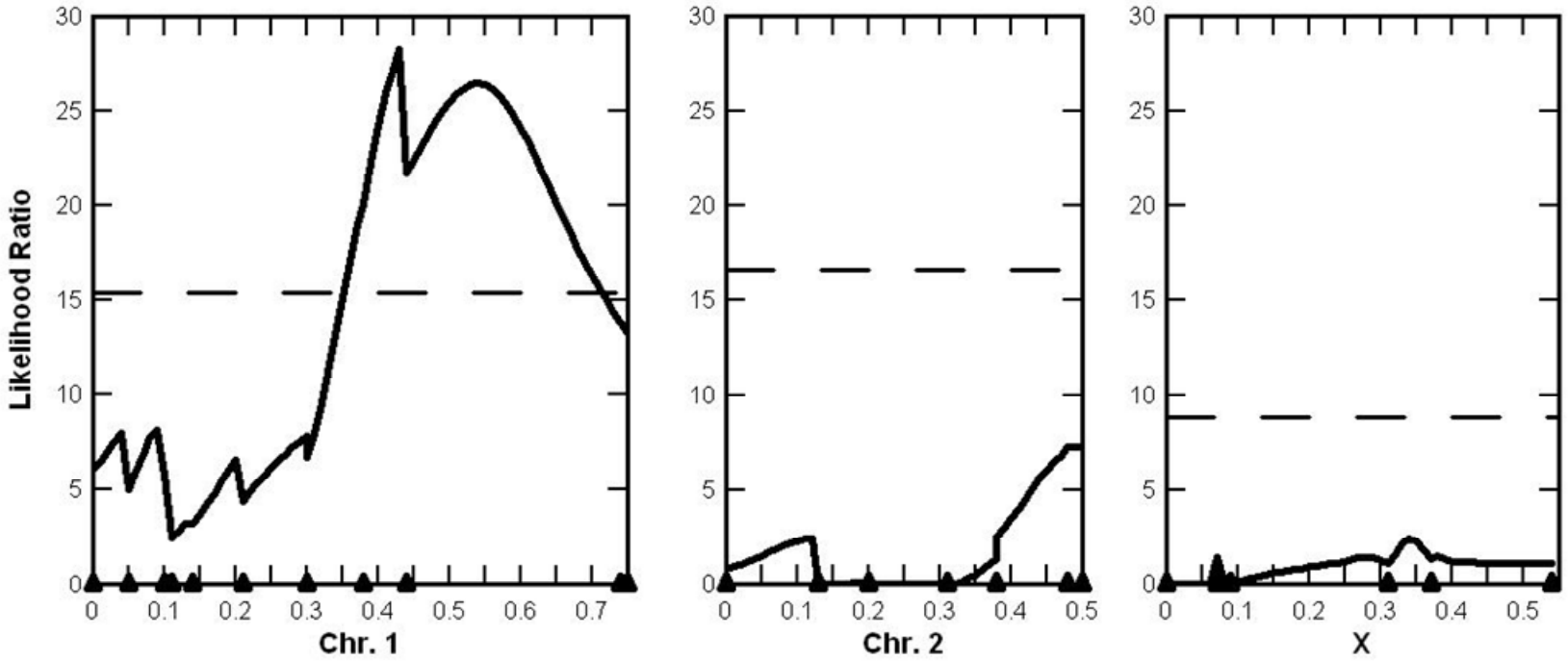

Progeny Sex Ratios
S(S(G^DS))
S(S(G^STS))
Unbiased
Biased
20
16
12
8
4
0
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1
Proportion male

Fig. 13


Likelihood Ratio
30
25
20
15
10
5
0
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7
Chr. 1
0 0.1 0.2 0.3 0.4 0.5
Chr. 2
0 0.1 0.2 0.3 0.4 0.5
X

Appendix

Supplemental Table 1 (Chapter 3): Sample sizes in each second-generation backcross family (Soraya backcrosses). Each male replicate is a separate male from the Soraya inbred line. In the female replicates, each male replicate was mated with either three or four full sisters from the pool of first generation backcross progeny. All females who yielded progeny used in the study are listed in this table.

cross	$F1^{D}xS$						$F1^{ST}xS$				
Male replicate	A	B			C		A	B			C
Female replicate	1	1	3	4	1	3	3	1	2	3	2
Number of sons	15	33	32	13	1	2	94	1	27	20	23

Supplemental Table 2 (Chapter 3): Sample sizes in each second-generation backcross family (Gombak backcrosses). Each male replicate is a separate male from the Gombak inbred line. In the female replicates, each male replicate was mated with either three or four full sisters from the pool of first generation backcross progeny. All females who yielded progeny used in the study are listed in this table.

cross	$F1^{D}xG$										$F1^{ST}xG$								
backcross replicate	A			B			C				A			B			C		
2nd-gen bx replicate	1	2	4	1	2	4	1	2	3	4	1	2	3	1	2	3	1	2	3
sample size	19	41	1	20	13	26	7	33	17	41	41	14	52	8	45	15	20	9	16

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